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Writer: Sunniva Lundal Haaland	Sunniva L. Haaland (signature of writer)
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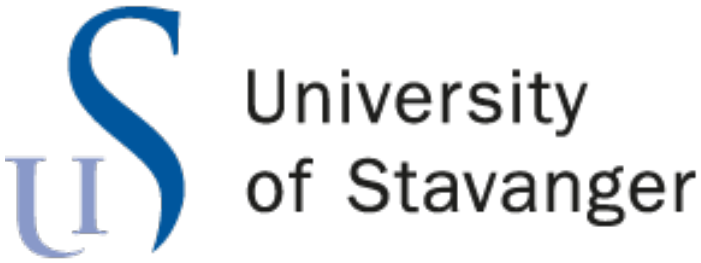

University of Stavanger – Faculty of Science and Technology

Master Thesis

Biological Chemistry, Master of Science Degree Program

Characterization of antimicrobial resistance in Norwegian blood culture isolates of *Klebsiella pneumoniae* using whole genome sequencing.

Supervisors:
Aasmund Fostervold
Eva Bernhoff

By
Sunniva Lundal Haaland

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Abstract

Antimicrobial drugs are used to treat and prevent infections caused by microorganisms. For decades, they have saved millions of lives, treated diseases and reduced pain. However, when exposed to antimicrobial drugs, microorganisms develop antimicrobial resistance (AMR) over time. This process is accelerated by the misuse and overuse of antibiotics. To handle this global health threat has become a high priority by WHO.

Klebsiella pneumoniae is a common intestinal bacterium that can cause life-threatening infections, such as pneumonia, wound, soft tissue or urinary tract infections. It is an important reservoir for a number of AMR-genes. *K. pneumoniae* can acquire extended-spectrum β-lactamase (ESBL) encoding genes which lead to resistance against broad-spectrum cephalosporins. *K. pneumoniae* is the 'K' in the ESKAPE pathogens, which is the six most significant and dangerous causes of drug-resistant hospital infections identified by the Infectious Diseases Society of America.

In this study, antimicrobial resistance determinants and presence of multidrug resistance in a population of 722 *K. pneumoniae* isolates were identified using Illuminas MiSeq WGS technology and several bioinformatics tools. It was taken particular interest in third-generation cephalosporins, aminoglycosides, fluoroquinolones, trimethoprim/ sulfamethoxazole and colistin. Detected resistance determinants were compared to detected phenotypical antimicrobial susceptibility, determined by micro broth dilution. This was done for a selection of isolates containing extended-spectrum β-lactamase genes or colistin resistance determinants.

K. pneumoniae sensu stricto was found to be the most prevalent species in this population of 722 isolates. A high diversity of sequence types (STs) was detected (n=378), where ST107 was the most prevalent (n=67).

20.5% (n=148) of this population had determinants encoding resistance to at least one of the drug classes investigated. Five isolates were found with genotypic colistin resistance due to truncations of PmrB and MgrB. $ESBL_A$-genes were detected in 50 isolates, where $bla_{CTX\text{-}M\text{-}15}$ (n=34) was the dominant $ESBL_A$-gene. ST307 containing $bla_{CTX\text{-}M\text{-}15}$ was the dominant $ESBL_A$ carrying sequence type (n=11).

46 isolates were found to be multidrug resistant, mainly to aminoglycosides, fluoroquinolones and trimethoprim/ sulfamethoxazole. However also strongly associated with carriage of $ESBL_A$, predominantly $bla_{CTX\text{-}M\text{-}15}$.

Based on the results, genotype cannot reliably predict phenotype for all the tested drug classes. However, presence of $ESBL_A$-genes coincides with phenotypic resistance against third-generation cephalosporins, indicating ability to predict a resistant phenotype.

Abbreviations

AMR - antimicrobial resistance

ESBL - Extended-spectrum β-lactamase

K. pneumoniae - *Klebsiella pneumoniae*

The Norwegian *Klebsiella* Bacteremiae study – NORKAB

MDR – multiple drug-resistant

TMP – Trimethoprim

SMX – Sulfamethoxazole

PBP – penicillin-binding protein

WGS – whole genome sequencing

NGS – next generation sequencing

ST – sequence type

MBD – micro broth dilution

AST – antimicrobial susceptibility testing

MIC – minimum inhibitory concentration

MLST – multi locus sequence typing

Kbp – kilo base pair

Mbp – mega base pair

Bp – base pair

NORM – Norwegian Surveillance System of Antibiotic Resistance in Microbes

Table of Contents

ACKNOWLEDGMENTS I

ABSTRACT II

ABBREVIATIONS IV

1. INTRODUCTION 1

1.1 BACKGROUND 1

1.2 *KLEBSIELLA PNEUNOMIAE* 2

1.2.1 Klebsiella pneumoniae species 2

1.3 ANTIBIOTICS 3

1.3.1 β-lactam antibiotics 3

1.3.2 Aminoglycosides 3

1.3.3 Fluoroquinolones 4

1.3.4 Trimethoprim/sulfamethoxazole 4

1.3.5 Colistin 4

1.4 ANTIMICROBIAL RESISTANCE 5

1.4.1 Development of antimicrobial resistance 5

1.5 EXTENDED-SPECTRUM B-LACTAMS (ESBLS) 5

1.5.1 Class A ESBL 7

1.6 EPIDEMIOLOGY OF ANTIBIOTIC RESISTANT *KLEBSIELLA PNEUMONIAE* 7

1.7 BACKGROUND METHODS 9

1.7.1 Illumina Sequencing 9

1.7.2 Antimicrobial susceptibility testing (AST) 12

2. AIMS OF STUDY 15

3. MATERIALS 16

3.1 INSTRUMENTS 16

3.2 COMMERCIAL KITS AND REAGENTS 17

3.3 COLLECTION OF BACTERIAL ISOLATES 18

4. METHODS 19

4.1 CULTIVATION OF BACTERIAL ISOLATES 19

4.2 DNA EXTRACTION 19

4.3 DNA CONCENTRATION MEASUREMENT AND NORMALIZATION 20

4.3.1 Concentration measurement 20

4.3.2 Normalization 21

4.4 NEXTERA XT LIBRARY PREPARATION FOR ILLUMINAS MISEQ 22

4.4.1 Perform a run using Hamilton ML Star 22

4.4.2 Tagmentation 23

4.4.3 Library amplification 23

4.4.4 Library Clean Up 23

4.4.5 Normalize Libraries 23

4.4.6 Pool Libraries 23

4.5 WHOLE GENOME SEQUENCING ON ILLUMINAS MISEQ 24

4.6 QUALITY MONITORING POST SEQUENCING 27

4.6.1 Quality control using MCS during sequencing 27

4.6.2 Quality assessment using Sequence Analysis Viewer (SAV) post sequencing 27

4.7 BIOINFORMATIC ANALYSIS OF SEQUENCE DATA 29

4.7.1 Quality check of raw data 30

4.7.2 Assembly 30

4.7.3 Quality valuation of genome assembly 31

4.7.4 Phylogenetic analysis *32*
4.7.5 Multi locus sequencing typing, species identification and resistance profiles *32*
4.8 ANTIMICROBIAL SUSCEPTIBILITY TESTING 33
4.8.1 Micro broth dilution *33*

5. RESULTS **35**
5.1 RAW DATA AND ASSEMBLY QUALITY 35
5.2 DISTRIBUTION OF SPECIES, SEQUENCE TYPES AND ESBL-HARBORING ISOLATES 36
5.2.1 Identification and distribution of Klebsiella pneumoniae species *36*
5.2.2 Phylogenetic analysis of the sequenced K. pneumoniae population, multi locus sequence typing and prevalence of ESBL genes *36*
5.2.3 Distribution of $ESBL_A$ genes in dominant $ESBL_A$-gene containing sequence types *37*
5.2.4 Geographical distribution of isolates with $ESBL_A$ encoding genes *38*
5.3 ANTIMICROBIAL RESISTANCE – DETECTION OF GENETIC AMR DETERMINANTS AND PHENOTYPICAL ANTIMICROBIAL SUSCEPTIBILITY TESTING 39
5.3.1a Detection of $ESBL_A$ encoding genes *39*
5.3.1b Investigation of imprecise $ESBL_A$ encoding genes allele matches *39*
5.3.2a Distribution of resistance determinants against important antimicrobial drug classes *41*
5.3.2b Resistance determinant gene variants *42*
5.3.3 Detection of multidrug resistance *43*
5.3.4 Comparison of phenotypic antimicrobial susceptibility testing with detection of resistance determinants *44*

6. DISCUSSION **49**
6.1 DISCUSSION METHODS 49
6.1.1 Next-generation Sequencing *49*
6.1.2 Antimicrobial susceptibility testing *49*
6.1.3 Bioinformatics analysis *50*
6.2 DISCUSSION RESULTS 51
6.2.1 Raw data and assembly quality *51*
6.2.2 Distribution of species and phylogenetic analysis *51*
6.2.3 Multi locus sequence typing, $ESBL_A$-harboring sequence types and geographic distribution of $ESBL_A$-genes *52*
6.2.4 Prevalence of known AMR-determinants *53*
6.2.5 Genetic characteristics of multidrug resistant isolates *54*
6.2.6 Comparison of phenotypic antimicrobial susceptibility testing with detection of resistance determinants *55*

7. CONCLUSION **58**

8. FUTURE PERSPECTIVES **59**

9. LIST OF REFERENCES **60**

APPENDIX A **67**

APPENDIX B **70**

APPENDIX C **72**

APPENDIX D **73**

1. Introduction

1.1 Background

Antimicrobial drugs are used as medicines to prevent and treat infections caused by microorganisms. [1] These medicines have saved many lives by treating infections and reduced pain. [2] When bacteria changes in response to these medicines, resistance to antimicrobial drugs occur. Misuse and overuse of antibiotics accelerate the process for microbes becoming resistant. Antimicrobial resistance (AMR) threatens the effective prevention and treatment of modern medicine to treat a range of infections caused by bacteria, parasites, viruses and fungi. [1], [3]

AMR is present in every country and is increasingly becoming a serious threat worldwide. New resistance mechanisms are evolving and spreading globally, which decrease the likelihood for effective treatment of antibiotics. [3] Handling AMR is a high priority for the World Health Organization. In May 2015, at the World Health Assembly the "Global action plan on antimicrobial resistance" was endorsed. The aim is to prevent and treat infectious diseases with effective and safe medicines. [1]

Klebsiella pneumoniae is a common intestinal bacterium that can cause life-threatening infections. [3] *K. pneumoniae* is the 'K' in the ESKAPE pathogens, which is the six most significant and dangerous causes of drug-resistant hospital infections identified by the Infectious Diseases Society of America. [4]

An increase in multidrug resistance (MDR) and production of extended-spectrum β-lactamase (ESBL), has led to the increased resistance against carbapenem antibiotics, which is used as a last-resort treatment of infections caused by MDR isolates. Colistin is the last resort treatment of carbapenem resistant *K. pneumoniae*. Recently, resistance to colistin have been detected in many countries, making infections caused by this bacterium untreatable. [3]

Whole genome sequencing is a method where entire genomes can be analyzed. The genomic information can be useful in providing knowledge about new resistance strains, dissemination of resistance and regulation mechanisms. [5], [6]

1.2 *Klebsiella pneunomiae*

Klebsiella pneumoniae is a Gram-negative, rod shaped bacterium belonging to the *Enterobacteriaceae* family. The bacterium is ubiquitous in the environment and can colonize in animals, plants and humans. [4] *K. pneumoniae* is an important reservoir for a number of AMR-genes and has been the leading cause of hospital-acquired infections and neonatal sepsis worldwide for many decades. [7] The bacterium is considered an opportunistic pathogen which can be carried asymptomatically in the nose, throat, intestinal tract and skin of healthy individuals. However, it can also cause a range of infections in hospitalized patients, most commonly pneumoniae, wound, soft tissue, or urinary tract infections. Especially elderly, neonates and immunocompromised are at risk. [5] Strains of *K. pneumoniae* is increasingly found to be resistant to multiple classes of antibiotics which makes it difficult to treat infections caused by this bacterium [8]. *K. pneumoniae* can acquire extended-spectrum β-lactamase (ESBL) encoding genes which lead to resistance against broad-spectrum cephalosporins. It was first reported in 1983 in Germany and has increased ever since. [9] Over the years, there has been an increase of multidrug resistant (MDR) *K. pneumoniae* strains, even including resistance to colistin (last line drug). This is identified as an urgent threat to the human health. [10] From 2012 to 2015 in Europe, combined resistance to fluoroquinolones, third- generation cephalosporins and aminoglycosides increased from 17.7% to 18.6%. [11]

1.2.1 *Klebsiella pneumoniae* species

Three phylogenetically distinct groups within the *Klebsiella pneumoniae* species, KpI, KpII and KpIII, have now been re-designated as distinct species, *K. pneumoniae, K. quasipneumoniae* and *K. variicola,* respectively. However, recent studies suggest that KpII should be further divided into the two subspecies *K. quasipneumoniae subs. quasipneumoniae* and *K. quasipneumoniae subs. similipneumoniae*, KpII-A and KpII-B respectively. All these can cause infections in humans. [4],[5],[12] In this thesis, the term *K. pneumoniae* will be used when referring to all species, KpI, KpII-A, KpII-B and KpIII, and *K. pneumoniae sensu stricto* when referring to KpI.

1.3 Antibiotics

In 1928, a new era in medicine was launched as Alexander Fleming discovered the first true antibiotic, penicillin [13]. Antibiotics have since then saved millions of lives, treated diseases and reduced pain, which have led to the drastically increase of the human life expectancy [2]. These compounds often act by interfering with different targets in the bacterial cell [14]. β-lactams, aminoglycosides, fluoroquinolones, trimethoprim/sulfamethoxazole and colistin are some of the most relevant groups of antibiotics regarding resistance in *Klebsiella pneumoniae* and the ones focused on in this study.

1.3.1 β-lactam antibiotics

β-lactam antibiotics are the most widely used antibiotics and includes pencillins, cephalosporins, monobactams, β-lactamase inhibitors and carbapenems. All β-lactam antibiotics have a β-lactam ring in their molecular structure [15], as shown in figure 1. By binding to penicillin-binding proteins and disrupting the synthesis of the bacterial peptidoglycan cell walls, these drugs are bactericidal. [16]. Production of β-lactamase, followed by alterations in cell wall permeability and extrusion of efflux pumps, are the first mechanism in resistance against these antibiotics in *K. pneumoniae*. [15]

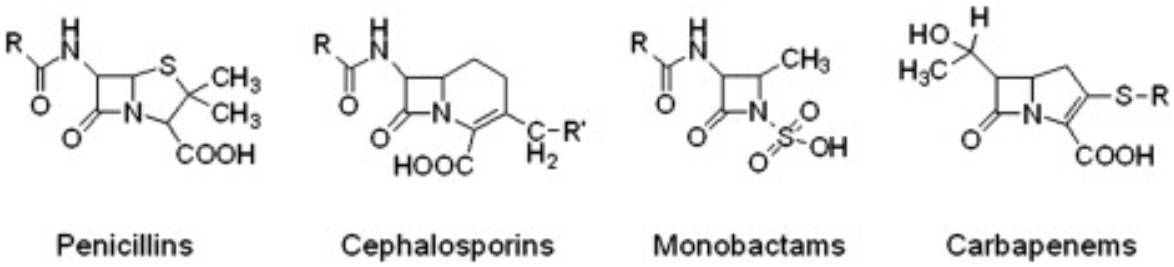

Figure 1: *Molecular structure of penicillins, cephalosporins, monobactams and carbapanems.* [17]

1.3.2 Aminoglycosides

Aminoglycosides are powerful, broad-spectrum antibiotics with many useful properties for the treatment of life-threatening infections caused by gram-negative bacteria. [18] They are bactericidal and act by inhibiting bacterial protein synthesis, where binding to receptors on the 30S subunit induce a misread of mRNA. [19] Decreased uptake and/or accumulation of the drug in bacteria, and the bacterial expression of enzymes which modify the antibiotic and thereby inactivate it, are the main mechanisms in resistance against aminoglycosides. [18]

1.3.3 Fluoroquinolones

Fluoroquinolones are broad-spectrum antibiotics used to treat infections caused by gram-positive and gram-negative bacteria [20]. They are the only direct inhibitors of the bacterial synthesis and by binding to the enzyme-DNA complex, they stabilize DNA strand breaks created by DNA gyrase (gyrA) and topoisomerase IV (ParC). [21] Additionally, mutations in the quinolone resistance-determining regions of gyrA and ParC genes, plasmid-mediated resistance to quinolones, altered permeability and lower uptake of drug are other mechanisms associated with fluoroquinolones resistance. [15]

1.3.4 Trimethoprim/sulfamethoxazole

The drug combination of Trimethoprim and Sulfamethoxazole (TMP/SMX) is a broad-spectrum antibiotic used for treating infections caused by both gram-negative and gram-positive organisms. It is widely used in treatment of many mild-to-moderate and more serious infections. [22] The drug is bactericidal and works by blocking the two steps in bacterial biosynthesis of essential nucleic acids and proteins. [23]

1.3.5 Colistin

Colistin is a 60-year-old antibiotic with significant activity against Gram-negative bacteria. It was revealed that colistin had severe side effects such as nephrotoxicity and neurotoxicity, and therefore the use of colistin was stopped and replaced by other antibiotics considered safe at the time. The increasing number of infections caused by multidrug resistant bacteria have led back to the use of colistin. [24] It is now being used as a ‘last-line drug’ to treat infections caused by multidrug resistant Gram-negative bacteria. [25] Colistin are bactericidal by disrupting the bacterial outer membrane resulting in cell death. [26] Recently, resistance to colistin has been detected in several countries. [3] In *K. pneumoniae*, resistance to colistin is related to lipopolysaccharide (LPS) modification following the addition of 4-amino-4-deoxy-L-arabinose to lipid A. [15] Recently, studies focus on the emergence of colistin resistance in multidrug resistant bacteria *K. pneumoniae* as a result of loss-of-function mutations of the MgrB gene and the discovery of mobile resistance (mcr) determinants. [10], [27]

1.4 Antimicrobial resistance

Antimicrobial resistance (AMR) is the ability of microorganisms to resist the effect when exposed to antimicrobial drugs. [3] Bacteria may acquire resistance by de novo mutations or acquire resistance genes from other organisms. [28] The process of bacteria becoming resistant is accelerated by the misuse and overuse of antimicrobials. Microbes that are antimicrobial resistant are indigenous in the environment and can be found in people, plants, animals and food. They can be spread by person-to-person contact, through food and between animals and humans. Proper food-handling, sufficient sanitary conditions, good hygiene and right infection control can slow down the spread of AMR. [3]

AMR threatens the modern medicine in a way it prevents and treats a range of infections caused by bacteria, parasites, fungi and viruses. Without effective antimicrobials, treatment and prevention of infections, surgeries and cancer chemotherapy, for example, would be at high risk due to less effective treatment with antibiotics. [3]

1.4.1 Development of antimicrobial resistance

Infections caused by antibiotic-resistant bacteria are harder to treat than those caused by non-resistant bacteria. [1] In bacteria, resistance can occur in different ways. Horizontal gene transfer allows genes to be inherited or acquired between different species of bacteria. Mutation can also lead to resistance. [29] Plasmids are small, mobile genetic elements which can add new properties to the bacteria such as resistance against antibiotics. [30]

1.5 Extended-spectrum β-lactams (ESBLs)

β-lactamases are enzymes that degrade the β-lactam ring. This results in a slightly different structure and inactivation of the drug, resulting in resistance to β-lactam antibiotics, such as penicillin and cephalosporins [31]. Extended-spectrum β-lactamases (ESBLs) are plasmid-enclosed enzymes that confer resistance on those β-lactams that were designed to resist such enzyme attack [32]. Plasmids responsible for ESBL production often carry resistance genes against other classes of antibiotics. [33]

β-lactamases can be classified into two major classification schemes, the Ambler and the Bush-Jacoby-Medeiros systems. These systems classify β-lactamases based on their enzyme structure (Ambler) and substrate profile, i.e., which class of β-lactams is degraded and to what

degree activity is inhibited by the β-lactamase inhibitor clavulanic acid (Bush-Jacoby-Medeiros). [34] An enzyme is classified as an ESBL if it is a molecular class A, and a functional class 2be enzyme. A classification was proposed by Giske et al. which expands the definition of ESBL to other clinically important acquired beta-lactamases with activity against extended-spectrum cephalosporins and/or carbapenems. In this classification scheme, ESBLs are categorized into three classes, $ESBL_A$, $ESBL_M$ and $ESBL_{CARBA}$ (table 1). The common ESBL families, CTX-M, TEM, SHV, PER and VEB, belongs in the $ESBL_A$ class. AmpC and OXA-ESBL are classified as the miscellaneous ESBLs ($ESBL_M$). Lastly, ESBLs with hydrolytic activity against carbapenems, the carbapanemases has been designated $ESBL_{CARBA}$. This class is further divided into $ESBL_{CARBA-A}$, $ESBL_{CARBA-B}$ and $ESBL_{CARBA-D}$ [35].

Table 1: *Proposal for classification of class A ESBLs ($ESBL_A$), miscellaneous ESBLs ($ESBL_M$) and ESBLs with hydrolytic activity against carbapenems ($ESBL_{CARBA}$). [35]*

<table>
<tr><td colspan="4">Acquired β-lactamases with hydrolytic activity against extended-spectrum cephalosporins and/or carbapenems</td></tr>
<tr><td rowspan="3">β-Lactamase classes</td><td>$ESBL_A$</td><td>$ESBL_M$</td><td>$ESBL_{CARBA}$</td></tr>
<tr><td>High prevalent $ESBL_A$
CTX-M
TEM-ESBLs
SHV-ESBLs
VEB
PER</td><td>$ESBL_{M-C}$ (Plasmid-mediated AmpC)
CMY
FOX
MIR
MOX
DHA
LAT
BIL
ACT
ACC</td><td>$ESBL_{CARBA-A}$
KPC
GES-2, -4, -5, -6, -8
NMC
SME
IMI-1, -2</td></tr>
<tr><td>Low prevalent $ESBL_A$
GES-1, -3, -7, -9
SFO-1
BES-1
BEL-1
TLA
IBC
CMT[a]</td><td>$ESBL_{M-D}$ (OXA-ESBL)
OXA-10-group
OXA-13-group
OXA-2-group
OXA-18
OXA-45</td><td>$ESBL_{CARBA-B}$ (MBL)
IMP
VIM
SPM-1
GIM-1
SIM-1
AIM-1

$ESBL_{CARBA-D}$ (OXA-carbapenemases)
OXA-23-group
OXA-24-group
OXA-48[b]
OXA-58-group</td></tr>
<tr><td>Operational definition</td><td>Non-susceptibility to extended-spectrum cephalosporins
AND
clavulanate synergy</td><td>Non-susceptibility to extended-spectrum cephalosporins
AND
phenotypic detection ($ESBL_{M-C}$)
OR
genotypic detection ($ESBL_{M-D}$)</td><td>Non-susceptibility to extended-spectrum cephalosporins and at least one carbapenem
AND
$ESBL_{CARBA}$ detected with phenotypic and/or genotypic methods</td></tr>
</table>

1.5.1 Class A ESBL

The most common enzymes in class A ESBL include SHV, TEM and CTX-M.
Since the 1980s, SHV-1 and TEM-2 were the predominantly ESBLs. Early 1990s, a new ESBL family emerged, called the CTX-M group.

In gram-negative bacteria, TEM-1 is the most frequently observed β-lactamase from the TEM family. This enzyme can hydrolyze penicillin and cephalosporins. TEM-3 was the first TEM-type β-lactamase that showed the ESBL phenotype. Most of the new derivatives are ESBLs, however some of these β-lactamases are inhibitor-resistant enzymes. [36]

In *K. pneumoniae* regarding the SHV family, SHV-1 β-lactamase is most commonly found. Production of SHV-1 makes *K. pneumoniae* resistant to ampicillin and carbenicillin. Most of SHV variants have an ESBL phenotype that are characterized by the substitution of a serine for glycine at position 238. Other variants, like those related to SHV-5, have a substitution of lysine for glutamate at position 240. The majority of SHV-type derivatives are ESBLs, but some have an inhibitor-resistant phenotype. [15], [36]

CTX-M hydrolyzes broad-spectrum oximino-β-lactams like cefotaxime, ceftriaxone and aztreonam and are easily inhibited by tazobactam and clavulanate. [37] CTX-M-type enzymes are rapidly spreading among *Enterobacteriaceae* and more than 200 allotypes are known. This rapid spread of these enzymes has made them the most prevalent ESBLs in *Enterobacteriaceae*, where $bla_{\text{CTX-M-15}}$ being the main enzyme currently encountered in *K. pneumoniae*. [15], [38]

1.6 Epidemiology of antibiotic resistant *Klebsiella pneumoniae*

Antibiotic resistance is present everywhere and can transfer from animals to humans, from food to humans, from person to person and through traveling. [39] Antibiotic resistance in *K. pneumoniae* is a public health concern. In the EARS-Net report for 2015, more than one third of the *K. pneumoniae* isolates were resistant to at least one of the antibiotic groups under surveillance (fluoroquinolones, third-generation cephalosporins, aminoglycosides and carbapenems). In figure 2, combined resistance to multiple antibiotic groups are shown. [11]

Many countries in eastern Europe stand out with 50–<75% of *K. pneumoniae* strains having combined resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides. Resistant isolates in Norway show a percentage of 1–<5%, which is significantly low compared to the rest of Europe. [40]

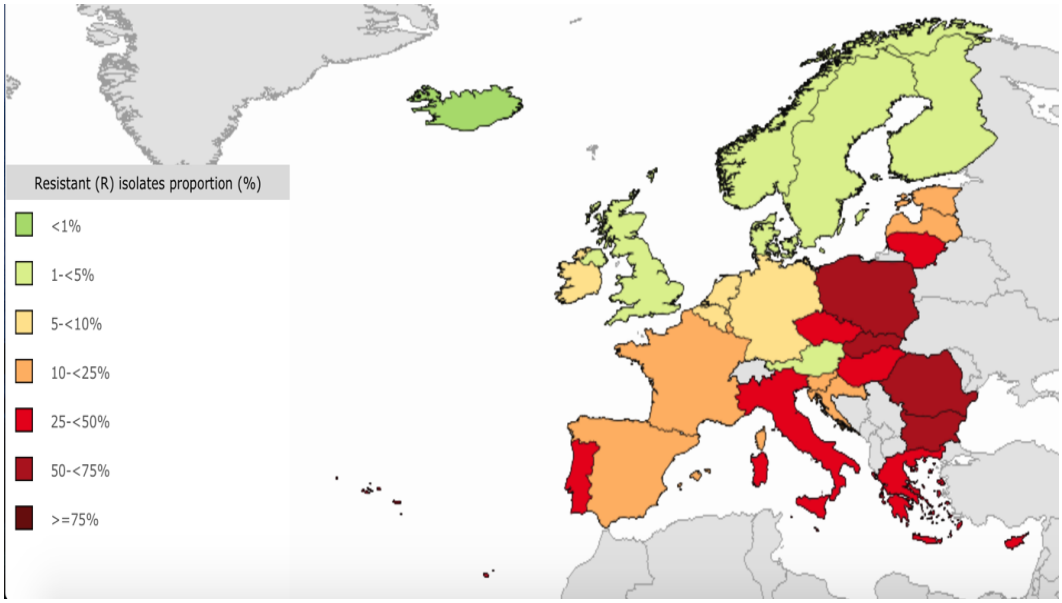

Figure 2: *Antimicrobial resistance of Klebsiella pneumoniae in Europe 2017. Percentage of invasive isolates with combined resistance to third-generation cephalosporins, fluoroquinolones and aminoglycoside. [40]*

However, NORM (Norwegian Surveillance System of Antibiotic Resistance in Microbes) reports an increasing amount of resistant *K. pneumoniae* since 2000, see figure 3. [41]

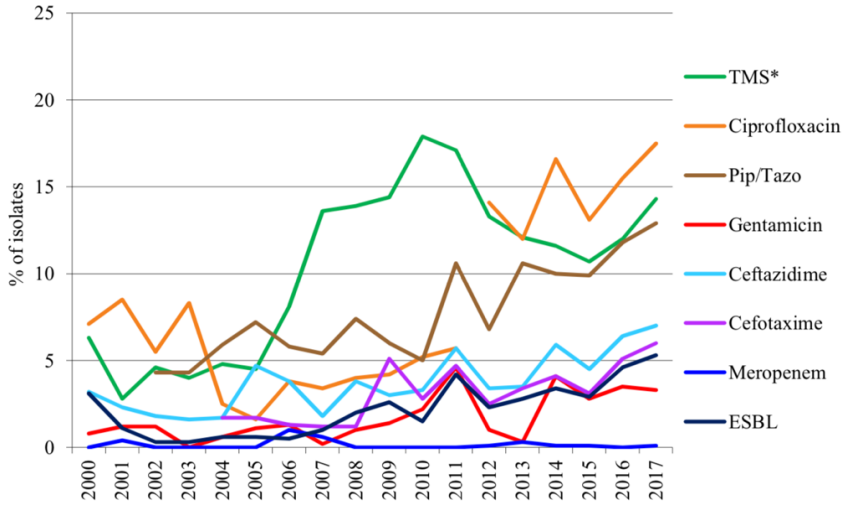

Figure 3: *Prevalence of non-susceptibility to various antimicrobial agents in Klebsiella pneumoniae blood culture isolates 2000- 2017. TMS*=Trimethoprim/ Sulfamethoxazole [41]*

1.7 Background Methods

DNA sequencing is used to determine the exact sequence of the nucleotides in a strand of DNA. Since the discovery of Sanger sequencing in 1977, DNA sequencing methods have evolved rapidly over the past decade in what are referred to next-generation sequencing (NGS) techniques. [42]

In both Sanger sequencing and NGS techniques, fluorescent nucleotides are added by DNA polymerase to a DNA strand, where each incorporated nucleotide is identified based on its fluorescent tag. The significant difference between Sanger sequencing and NGS techniques is the number of DNA fragments that can be sequenced at a time. Sanger sequencing are often used for small-scale projects, where the method only sequences a single DNA fragment at a time, while NGS can sequence millions of DNA fragments simultaneously per run. Illumina holds the main part of the NGS marked, although there are some other suppliers. In this thesis, Illuminas MiSeq was used to perform sequencing. [43]

1.7.1 Illumina Sequencing

The workflow on Illumina MiSeq includes four basic steps: 1. Library preparation, 2. Cluster generation, 3. Sequencing and 4. Data analysis.

Nextera XT DNA library preparation protocol, described in this thesis, is the procedure recommended for whole genome sequencing (WGS) of bacterial genomes on Illumina. Other procedures are recommended when sequencing mixed bacterial content or human DNA.

1.7.1.1 Library Preparation

Sample preparation starts with extracted and purified DNA, with a concentration of 0,2 ng/µl. During the first process called tagmentation, transposomes cut and tag the DNA fragments with adapter sequences. Through library amplification, two sets of unique index sequences are added to each DNA fragment in a process called multiplexing. Libraries with unique indexes can be pooled together and sequenced in the same run. Each read can be identified by the flow cell based on the unique index sequences. P7 and P5 are complementary to the lawn of oligos represented on the surface of the flow cell. In figure 4, an illustration of tagmentation and library amplification is shown. [44], [45]

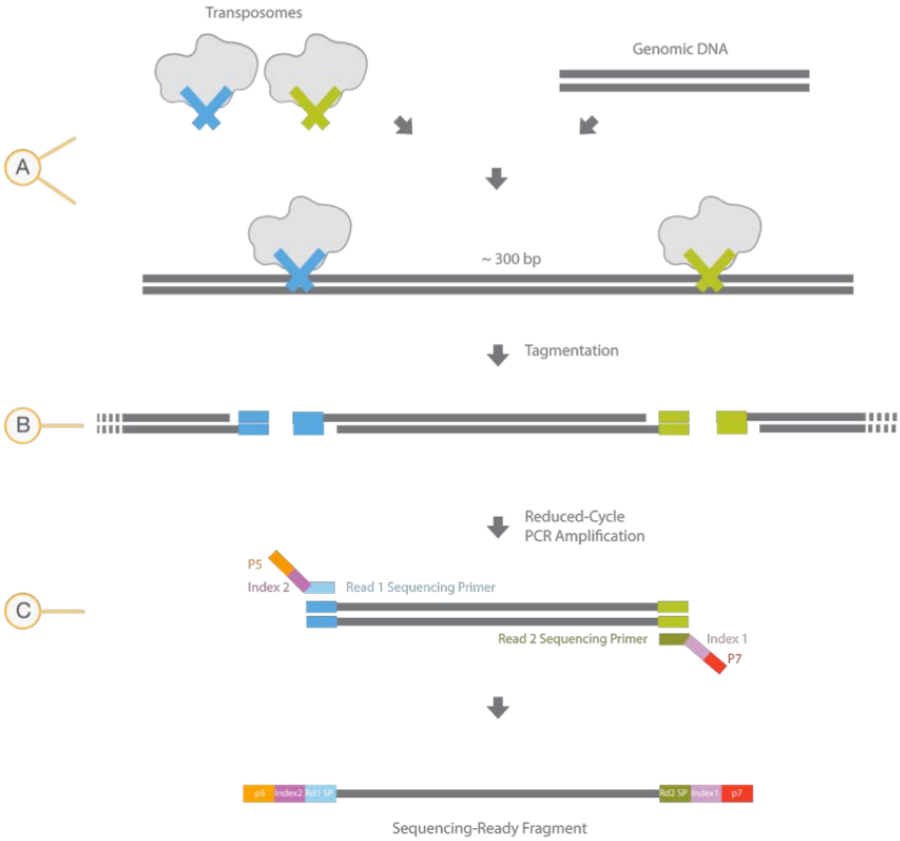

Figure 4: *Step A and B – Tagmentation: transposomes cut and attach adapters to the genomic DNA. Step C – Library amplification: two sets of unique index sequences are attached to the adapters on each end of the DNA fragment forming a DNA fragment ready to be sequenced.* [46]

1.7.1.2 Cluster generation by bridge amplification

Clustering is a process where the library is loaded onto the flow cell, a glass plate with lanes coated with two types of oligos. These oligos are complementary to the DNA library fragments and by hybridization the DNA strands are attached to the flow cell surface. A complement strand of the hybridized fragment is created by a polymerase, and the double stranded molecule is denatured where the original template is washed away. Clusters of 1000 identical DNA copies is formed for each strand through bridge amplification. In this process, the strand flips and forms a bridge by hybridizing to the second type of oligo on the flow cell, shown in figure 5.

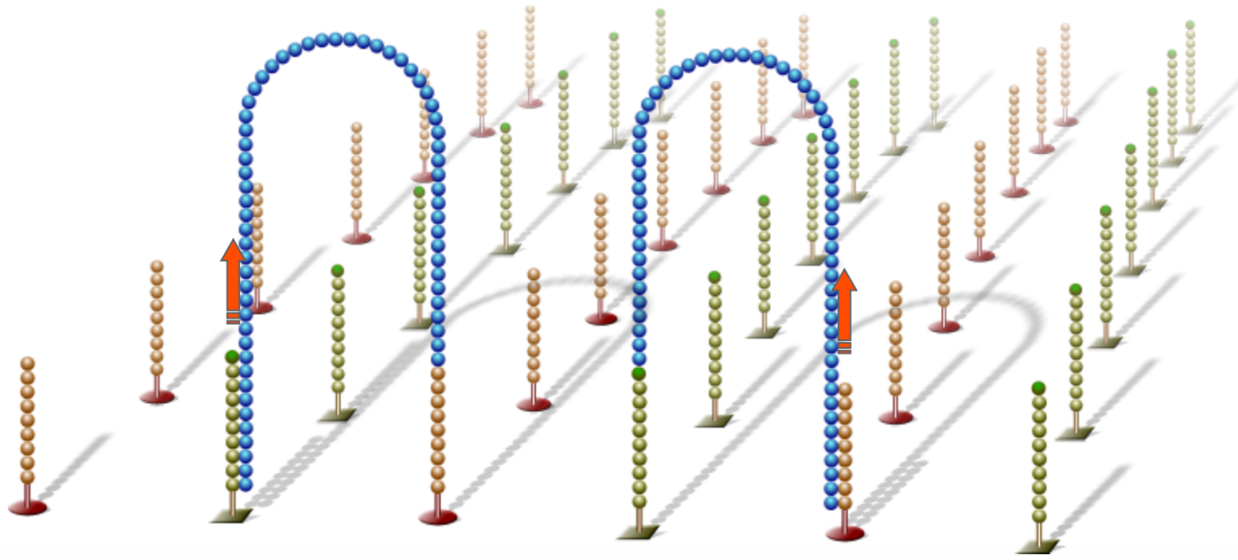

Figure 5: *Bridge amplification creating clonal clusters. Single-stranded DNA molecules bends over and are attached to oligos on the flow cell to form a bridge. [47]*

A polymerase extends the hybridized primer forming a double stranded bridge. This bridge is denatured resulting in two copies of covalently bound single stranded DNA templates. Then, the reverse strands are cleaved and washed off, leaving only the forward strands. The free 3'-ends of the forward strands are blocked to prevent unwanted priming. [44]

1.7.1.3 Sequencing by synthesis

Sequencing is a process where a sequencing primer is introduced to the flow cell and is hybridized to the adapter sequence annealing site. Fluorescently tagged nucleotides (dNTPs) are added together with a polymerase. The dNTPs have a terminator that prevents the polymerase of adding another dNTP. After the addition of the nucleotides the clusters are excited by a light source and a unique fluorescent signal is emitted and detected by the MiSeq instrument. After color detection, the terminator and fluorophore are cut off allowing the addition of a new base call. The length of the read is determined by the number of cycles, which is the incorporation of a nucleotide, base detection and fluorophore/ terminator cleavage. The base call is determined by the emission wavelength and the signal intensity. In a massively parallel process called Sequencing by synthesis technology, hundreds of millions of clusters are sequenced, shown in figure 6.

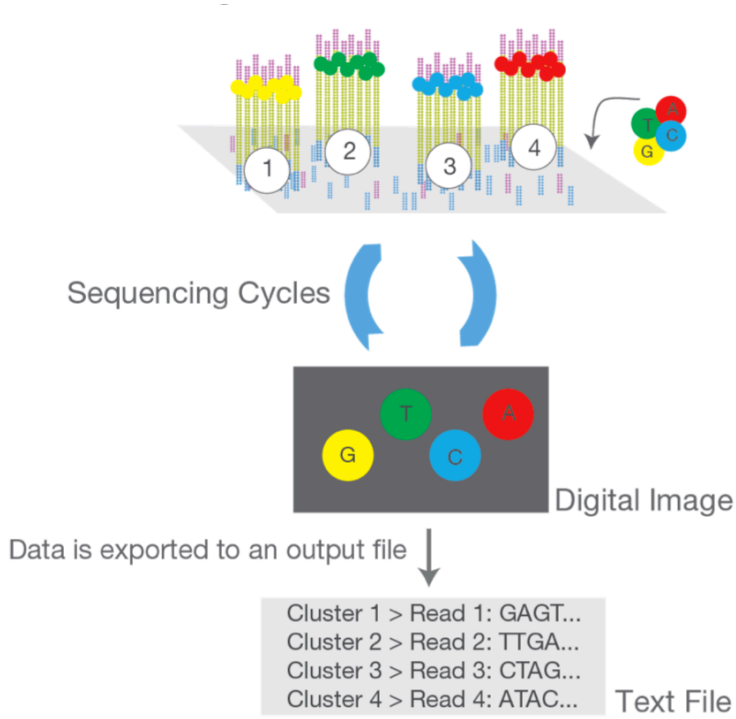

Figure 6: *Sequencing by synthesis technology uses four fluorescent dNTP added together with a polymerase. The clusters are excited by a light source and a unique fluorescent signal is emitted and detected by the MiSeq instrument, which provides a digital image. Data is exported to a text file where a sequence for each read is given. These sequences are further analyzed and aligned during data analysis.*

The Illumina MiSeq system provides paired-end sequencing. Meaning that after the first read is completed, the read 1 product is washed away. Then, the index 1 primers are hybridized to the template, and after the index read, the product is washed away. It binds to the second flow cell oligo by flipping over. Then index 2 is read similarly to index 1. A double stranded bridge is created by a polymerase and are denatured leaving only the reverse strands. Read 2 begins, and for both reads, the sequencing steps are repeated until preferred length is achieved. Then the read 2 product is washed away. [47], [44], [45]

1.7.2 Antimicrobial susceptibility testing (AST)

Antimicrobial susceptibility testing (AST) is performed to determine which antibiotics the bacteria is sensitive to. The information provided from these tests are important to guide antibiotic treatment decisions and predict the outcome. [48] In this thesis, micro broth dilution (MBD) was performed and the results was compared to the detected resistance genes in the dataset provided from sequencing.

1.7.2.1 Micro broth dilution

Micro broth dilution is a method used to determine the susceptibility to antibiotics of a bacterial isolate. Mueller-Hinton broth suspension with bacteria is inoculated to plates. Each plate is dosed with antimicrobial agents at appropriate dilutions. All plates include one positive control well where growth is required for the tests to be valid. After the plates have been incubated, quantitative minimum inhibitory concentration (MIC) results can be determined based on the actual growth of bacteria. The bacterial isolates are categorized qualitatively as either intermediate (I), susceptible (S) or resistant (R) to the different types of antibiotics. [49]

This method was performed using a nephelometer, an automated inoculation delivery system, a digital MIC viewing system along with SWIN software. The MIC-values were interpreted according to MIC breaking points provided by European Committee of Susceptibility Testing (EUCAST). Sensititre NONAG5 and Sensititre NONAG4, shown in figure 7 and 8 respectively, were the plates used in this method. These plates are well suited for testing of *Klebsiella pneumoniae* and other Gram-negative bacteria, based on the antimicrobial agents dosed in these plates.

1910161533

SENSITITRE
NONAG5
Standard Reference Card

	1	2	3	4	5	6	7	8	9	10	11	12
A	CIP 0.015	CIP 4	CZA 0.5/4	AMI 2	TOB 0.5	TOB 128	GEN 16	NIT 128	FOS+ 2	DOR 0.03	SXT 0.12/2.38	TGC 0.12
B	CIP 0.03	CIP 8	CZA 1/4	AMI 4	TOB 1	TOB 256	GEN 32	NIT 256	FOS+ 4	DOR 0.06	SXT 0.25/4.75	TGC 0.25
C	CIP 0.06	CIP 16	CZA 2/4	AMI 8	TOB 2	GEN 0.25	GEN 64	COL 0.25	FOS+ 8	DOR 0.12	SXT 0.5/9.5	TGC 0.5
D	CIP 0.12	CIP 32	CZA 4/4	AMI 16	TOB 4	GEN 0.5	GEN 128	COL 0.5	FOS+ 16	DOR 0.25	SXT 1/19	TGC 1
E	CIP 0.25	CZA 0.03/4	CZA 8/4	AMI 32	TOB 8	GEN 1	GEN 256	COL 1	FOS+ 32	DOR 0.5	SXT 2/38	TGC 2
F	CIP 0.5	CZA 0.06/4	CZA 16/4	AMI 64	TOB 16	GEN 2	NIT 16	COL 2	FOS+ 64	DOR 1	SXT 4/76	TGC 4
G	CIP 1	CZA 0.12/4	CZA 32/4	AMI 128	TOB 32	GEN 4	NIT 32	COL 4	FOS+ 128	DOR 2	SXT 8/152	TGC 8
H	CIP 2	CZA 0.25/4	AMI 1	AMI 256	TOB 64	GEN 8	NIT 64	COL 8	DOR 0.015	DOR 4	SXT 16/304	POS

ANTIMICROBICS

CIP	Ciprofloxacin
CZA	Ceftazidime/avibactam
AMI	Amikacin
TOB	Tobramycin
GEN	Gentamicin
NIT	Nitrofurantoin
COL	Colistin
FOS+	Fosfomycin+glucose-6-phosphate
DOR	Doripenem
SXT	Trimethoprim / sulfamethoxazole
TGC	Tigecycline
POS	Positive Control

Figure 7: *NONAG5 plate dosed with antimicrobial agents at appropriate dilutions.* [50]

2011140914

SENSITITRE

NONAG4

Standard Reference Card

	1	2	3	4	5	6	7	8	9	10	11	12
A	MERO	MERO	IMI	IMI	ETP	ETP	TAZ	FEP	P/T4	FOT	AZT	TRM
	0.015	4	0.06	16	0.015	4	16	4	1/4	0.12	0.12	4
B	MERO	MERO	IMI	IMI	ETP	TAZ	TAZ	FEP	P/T4	FOT	AZT	TRM
	0.03	8	0.12	32	0.03	0.12	32	8	2/4	0.25	0.25	8
C	MERO	MERO	IMI	AUGC	ETP	TAZ	FEP	FEP	P/T4	FOT	AZT	TRM
	0.06	16	0.25	4/2	0.06	0.25	0.06	16	4/4	0.5	0.5	16
D	MERO	FOX	IMI	AUGC	ETP	TAZ	FEP	FUR	P/T4	FOT	AZT	TRM
	0.12	2	0.5	8/2	0.12	0.5	0.12	1	8/4	1	1	32
E	MERO	FOX	IMI	AUGC	ETP	TAZ	FEP	FUR	P/T4	FOT	AZT	TRM
	0.25	4	1	16/2	0.25	1	0.25	2	16/4	2	2	64
F	MERO	FOX	IMI	AUGC	ETP	TAZ	FEP	FUR	P/T4	FOT	AZT	TRM
	0.5	8	2	32/2	0.5	2	0.5	4	32/4	4	4	128
G	MERO	FOX	IMI	AUGC	ETP	TAZ	FEP	FUR	P/T4	FOT	AZT	TRM
	1	16	4	64/2	1	4	1	8	64/4	8	8	256
H	MERO	FOX	IMI	AUGC	ETP	TAZ	FEP	FUR	FOT	FOT	AZT	POS
	2	32	8	128/2	2	8	2	16	0.06	16	16	

ANTIMICROBICS

MERO	Meropenem
FOX	Cefoxitin
IMI	Imipenem
AUGC	Amoxicillin / clavulanic acid constant 2
ETP	Ertapenem
TAZ	Ceftazidime
FEP	Cefepime
FUR	Cefuroxime
P/T4	Piperacillin / tazobactam constant 4
FOT	Cefotaxime
AZT	Aztreonam
TRM	Temocillin
POS	Positive Control

Figure 8: *NONAG4 plate dosed with antimicrobial agents at appropriate dilutions. [51]*

2. Aims of study

The main aim of this study is to characterize and define the AMR genes of human blood culture isolates of *K. pneumoniae* in Norway between 2010 and 2015.

Research questions:

- What is the prevalence of known AMR-genes in a population of 1000 blood culture isolates?
- What are the genetic characteristics of a selected number of multidrug resistant isolates?
- How does presence or absence of genetically detected antimicrobial resistance determinants correlate with results of phenotypic antibiotic susceptibility testing using relevant methods?

3. Materials

3.1 Instruments

Instruments used in this thesis were selected based on the specific methods, see table 2.

Table 2: *Instruments used in this thesis to perform following methods. Instruments in green are used to perform sequencing, while instruments in grey are used to perform micro broth dilution.*

Instrument	Function	Supplier	City, country
MiSeq [52]	High performance sequencer	Illumina	San Diego, CA, USA
Hamilton ML Star [53]	Used for automated pipetting of liquid sample material and reagents.	Hamilton	Giarmata, Timis County, Romania
MagNA Pure 96 [54]	High-throughput robotic workstation for fully automated purification of nucleic acids from up to 96 samples.	LifeScience Roche	Basel, Switzerland
Spark® [55]	Multimode microplate reader	Tecan	Mannedörf, Switzerland
Sensititre™ Nephelometer [56]	Standardize inoculum density	Thermo Fischer Scientific	Waltham, MA, USA
Sensititre AIM™ Automated Inoculation Delivery System [57]	Doses quickly and accurately 96-well plates	Thermo Fischer Scientific	Waltham, MA, USA
Sensititre™ Vizion™ Digital MIC Viewing System [58]	Automated read of visual results	Thermo Fischer Scientific	Waltham, MA, USA
Sensititre™ SWIN™ Software Epidemiology Module [59]	Generate complete, real-time reports and bar graphs in just minutes	Thermo Fischer Scientific	Waltham, MA, USA

3.2 Commercial kits and reagents

Different commercial kits were used for different procedures and machines. In table 3, an overview of the kits used during this study is listed.

Table 3: *An overview of commercial kits used in this study. Kits in green are used to perform sequencing, while kits in grey are used to perform micro broth dilution.*

Commercial kit	Function	Supplier	City, country
MagNAPure 96 DNA and Viral NA Small Volume Kit [54]	Purifies DNA using magnetic glass particle technology.	LifeScience Roche	Basel, Switzerland
Quant-iT™ dsDNA assay kit, high sensitivity [60]	DNA quantification.	ThermoFisher Scientific	Waltham, MA, USA
Nextera XT DNA Library Preparation Kit [61]	Prepare sequencing libraries for small genomes, PCR amplicons, plasmids or cDNA.	Illumina	San Diego, CA, USA
PhiX Control V3 [62]	Control library for Illumina sequencing runs.	Illumina	San Diego, CA, USA
MiSeq Reagent Kit V3 [63]	Sequencing. Enable the highest output of all MiSeq kits.	Illumina	San Diego, CA, USA
Sensititre NONAG4 [51]	Plate dosed with antimicrobial agents at appropriate dilutions. Used when performing antimicrobial susceptibility test.	Thermo Fischer Scientific	Waltham, MA, USA
Sensititre NONAG5 [50]	Plate dosed with antimicrobial agents at appropriate dilutions. Used when performing antimicrobial susceptibility test.	Thermo Fischer Scientific	Waltham, MA, USA

3.3 Collection of bacterial isolates

Through the Norwegian *Klebsiella pneumoniae* bacteremia study (NORKAB), 722 *K. pneumoniae* isolates from 17 hospitals in Norway were selected for investigation. *K. pneumoniae* NORKAB collected blood culture isolates from adult patients from September 2018 to January 2019. In table 4, an overview of isolates from each hospital is given.

NORKAB investigate the relationship between *K. pneumoniae* genome, disease severity and outcome, and patient characteristics in patients with *K. pneumoniae* bacteremia.

Table 4: *Number of isolates from different hospitals obtained in this study.*

	Hospitals	Region	Isolates
	Akershus University Hospital	Oslo/ Akershus	97
	Sørlandets Hospital	South	12
	Nordland Hospital HF	North	4
	Diakonhjemmet	West	21
	Vestre Viken HF	East	46
	Hospital Østfold	East	11
	Finnmark Hospital HF	North	4
	The University Hospital Nord-Norge	North	19
	Haukeland University Hospital	West	97
	Hospital Innlandet	East	65
	Førde Hospital Trust	West	15
	Rikshospitalet	Oslo/ Akershus	42
	St. Olav's Hospital	Middle	39
	Stavanger University Hospital	West	82
	Telemark Hospital	South	46
	Vestfold Hospital	South	69
	Oslo University Hospital	Oslo/ Akershus	53
Total	17 hospitals		722

4. Methods

4.1 Cultivation of bacterial isolates

Protocol:

Microbank freezing vials containing *Klebsiella pneumoniae* were collected from the -80°C freezer. Blood agar plates were labeled with name, number and barcode. A glass bead/magnet was collected from the Microbank vial and transferred to the blood agar plate using an inoculating loop. The bead was streaked on the plate in a three-dilution pattern as shown in figure 9. The blood plates were incubated overnight at 35°C.

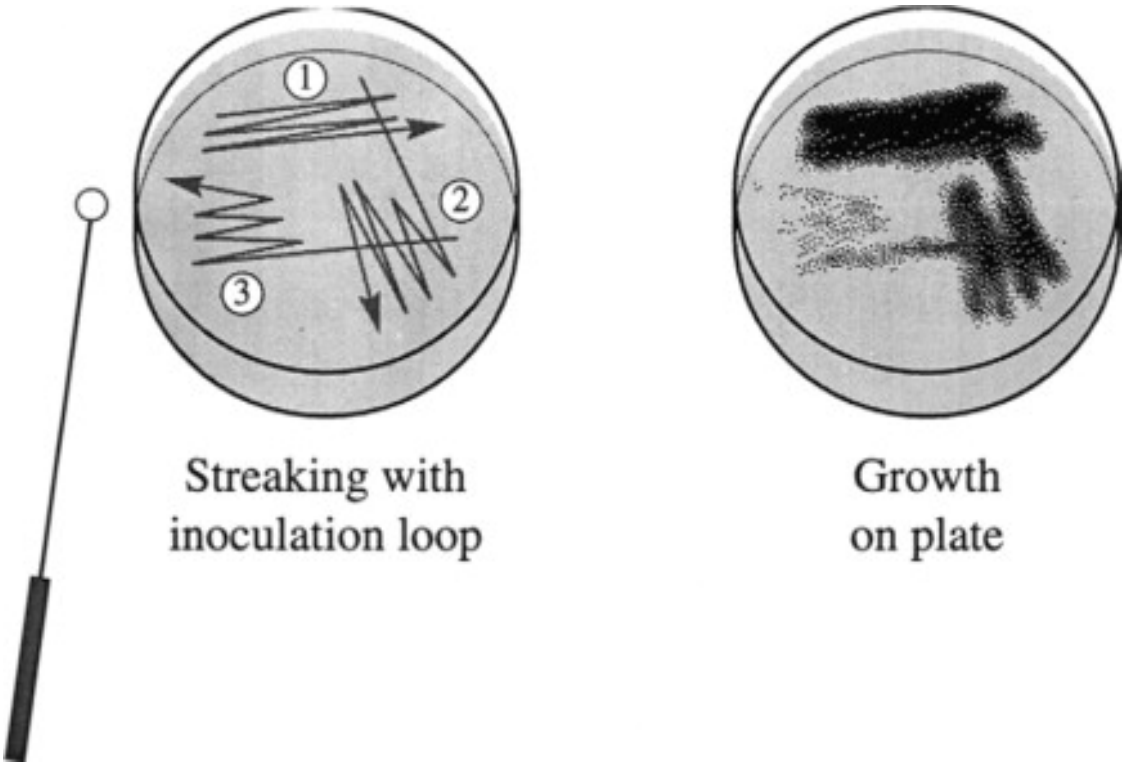

Figure 9: *Method for streaking single colonies using an inoculation loop. Numbered lines show the order and direction of streaking with the inoculation loop. The plate to the right shows growth of bacteria after incubation overnight*. [64]

4.2 DNA extraction

The bacterial DNA must be extracted before sequencing the genome of the isolates of *K. pneumoniae*. To extract DNA, MagNA Pure 96 System is used. The MagNA Pure 96 System purifies DNA, RNA, and viral nucleic acids from a wide range of starting materials using magnetic glass particle technology [54].

Protocol for extracting DNA:

A few colonies of *Klebsiella pneumoniae* were taken out from the overnight blood agar plate and transferred to an Eppendorf tube containing 500 µl of sterile saltwater using an inoculation loop. The tubes were vortexed and 200 µl of each sample were transferred to separate wells in a MagNA Pure Processing Cartridge. MagNA Pure 96 DNA and Viral NA Small Volume with the Pathogen Universal 200 3.1 protocol was used [65].

4.3 DNA concentration measurement and normalization

All samples must be normalized to the same concentration of 0.2 ng/µl before initiating DNA library preparation.

4.3.1 Concentration measurement

To determine the exact concentration of extracted DNA from the *K. pneumoniae* isolates, TECAN Spark (2) was used together with Quant-iT^{TM} dsDNA assay high sensitivity kit (1) [66]. Due to high concentrations, the extracted DNA was diluted 1:2, in 10 mM Tris-HCl, pH 7.5, before measuring concentration.

1. Quant-iT^{TM} dsDNA assay high sensitivity kit:

1. The assay components were equilibrated to room temperature.
2. A working solution was prepared by diluting the Quant-iT^{TM} dsDNA HS reagent 1:200 in Quant-iT^{TM} dsDNA HS buffer. For 32 samples (+ 2 standards), 10.4 ml of working solution was made (10.348 ml buffer + 52 µl fluorescent reagent) in a Falcon tube covered with tin foil to prevent daylight from disturbing the fluorescent solution.
3. 200 µl of working solution was loaded to each microplate well.
4. The Quant-iT^{TM} dsDNA HS standards were vortexed and centrifuged before adding 10 µl of each standard to separate wells.
5. 10 µl of each unknown DNA sample was added to separate wells.
6. The microplate was sealed and then placed in the shaker for 1 min at 1400 rpm.

2. Measuring DNA concentration using TECAN Spark:

1. The computer and monitor were switched on.
2. The Magellan Spark Control icon was clicked to open the program. The slider opens and the microplate with samples were placed here.
3. From the menu; **create a sample ID list** was selected. All sample IDs were scanned or typed in. The file was saved on the computer.
4. From the main menu; **Start measurement** was selected. A method used for Quant-iT^{TM} dsDNA assay high sensitivity kit, called High-Sensitivity was chosen. The correct sample ID list was selected and the method was started.
5. It resulted in a graph and calculated concentration for each DNA sample. This file was saved as a comma-separated values (CSV) file.

4.3.2 Normalization

Hamilton Microlab STAR (ML STAR) was used for automated pipetting of liquid sample material and reagents. [53] The concentration of each sample can be adjusted to 0.2 ng/µl using the program called Normalization on Hamilton.

Perform normalization using Hamilton ML STAR:

1. Hamilton ML STAR and cooling element were turned on.
2. The instrument control computer was turned on.
3. From the ML STAR desktop, Hamilton App Launcher was opened.
4. The program called **Normalization** was selected.
5. The CSV file from previous method was inserted as a file.
6. The on-screen instructions were followed to load the ML STAR carries. **OK** was clicked after loading the carriers.
7. **OK** was clicked to verify all labware positions and the run began.

4.4 Nextera XT library preparation for Illuminas MiSeq

The workflow for Nextera XT library preparation is shown in figure 10. Performing Nextera XT on Hamilton is Illumina qualified.

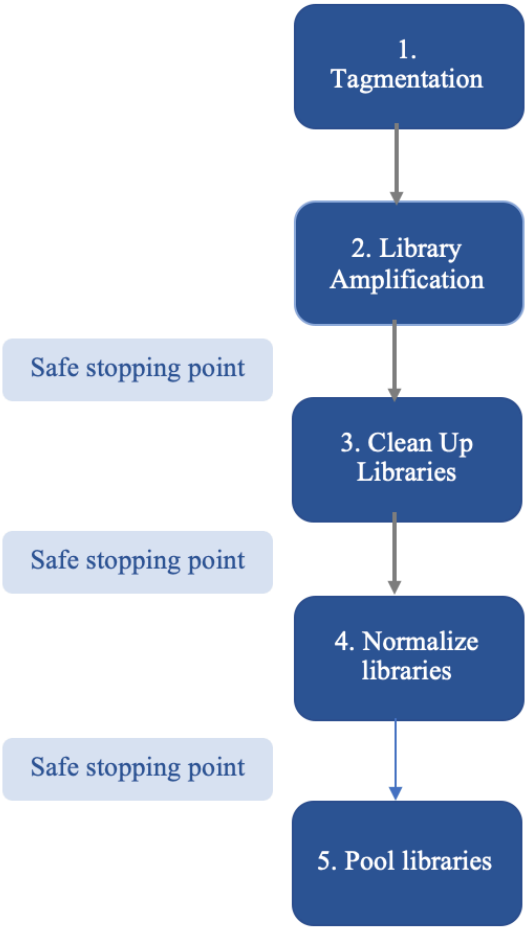

Figure 10: *Nextera XT workflow. Safe stopping points are marked between steps.*

4.4.1 Perform a run using Hamilton ML Star

1. Hamilton ML STAR, cooling element and instrument control computer were turned on.
2. From the ML STAR desktop, Hamilton App Launcher was opened and the appropriate program, Nextera XT, and the appropriate method, tagmentation, library amplification, clean up libraries, normalization or pooling, was selected.
3. The on-screen instructions were followed to load the ML STAR carries. **OK** was clicked after loading the carriers.
4. **OK** was clicked to verify all labware positions and the run began.

4.4.2 Tagmentation

This step used the Nextera transposome to tagment gDNA, which is a process that fragments DNA and then tags the DNA with adapter sequences in one step. [67] Tagmentation was performed using Hamilton (as described in 4.4.1), details about the procedure can be found in appendix A.

4.4.3 Library amplification

In this step, the tagmented DNA was amplified using a limited-cycle PCR program. The Index 1 (i7), Index 2 (i5), and full adapter sequences were added to the tagmented DNA by PCR. The adapters and indexes were required for cluster formation. [67] Library amplification was performed using Hamilton (as described in 4.4.1), details about the procedure can be found in appendix A.

4.4.4 Library Clean Up

In this step, AMPure XP beads was used to purify the library DNA and remove short library fragments. [67] Library clean-up was performed using Hamilton (as described in 4.4.1), details about the procedure can be found in appendix A.

Agilent Technology 2100 Bioanalyzer was used to check the libraries. Agilent High Sensitivity DNA Kit Guide was the protocol used. [68]

4.4.5 Normalize Libraries

The quantity of each library is normalized in this process to ensure more equal library representation in the pooled library. [67] Normalization was performed using Hamilton (as described in 4.4.1), details about the procedure can be found in appendix A.

4.4.6 Pool Libraries

Pooling libraries combine equal volumes of normalized libraries in one tube. [67] Pooling was performed using Hamilton (as described in 4.4.1), details about the procedure can be found in appendix A.

4.5 Whole Genome Sequencing on Illuminas MiSeq

Some preparations must be done before the pooled DNA libraries can be sequenced on Illuminas MiSeq. This included diluting and denaturation of the pooled libraries, preparation of the reagent cartridge and the set-up of a run using MiSeq Control Software (MCS). MiSeq Reagent Kit V3, 600 cycles, was used (MS-102-3003, Illumina).

Denaturing and Diluting Libraries and PhiX Control for Sequencing:

1. The incubator was preheated to 98^{o}C
2. 15 µl of the pool was combined with 585 µl of HT1 buffer in an Eppendorf tube. The tube was centrifuged at 280 x g at 20^{o}C for 1 minute.
3. The tube was placed on the incubator for 2 minutes. It was put immediately put on ice for 5 minutes (or until you are ready to load libraries on to the cartridge).
4. 6 µl of 12 pM PhiX Control was added to the libraries.

Preparing the Reagent Cartridge:

1. The cartridge was taken out from the freezer the day before use. One may also thaw the reagent cartridge in water bath for an hour.
2. The reagent cartridge was inverted then times to mix the thawed reagents and it was visually examined that all reagents had been thawed.
3. The foil, labeled **Load Samples**, on the cartridge was pierced with a 1 ml clean pipette. The total volume of libraries and PhiX (606 µl) were loaded onto the reagent cartridge without touching the foil.

Set Up a Run Using MiSeq Control Software (MCS):

1. From the Home screen, Manage Instrument was selected. A reboot was performed to restart the system.
2. A sample plate was made in the Illumina Experiment Manager (IEM) program.
    a. From the IEM main screen; **Create Sample Plate** was selected.
    b. Index kit **NEXTERA XT Index kit** was selected (24 indexes, 96 samples)
    c. The plate was given a unique plate name. (e.g. Date_initials)
    d. Index scheme was selected – **2 – libraries are dual index**.
    e. Plate tab was selected. This mimic the layout of a 96-well plate with columns A-H, rows 1-12.

f. Sample ID was scanned or typed in along with the correct indexes (each sample was given a unique pair of indexes).
g. Finish was selected and the plate was saved.

3. A sample sheet was made in the IEM program.
    a. From the IEM main screen; **Create Sample Sheet** was selected.
    b. **MiSeq** was selected.
    c. An appropriate application was selected – Fastq only
    d. In the Reagent Kit Barcode the reagent kit ID from the reagent cartridge was entered.
    e. Index adapter was selected – **Nextera XT index kit (24 indexes, 96 samples)**
    f. Index reads, **2 – libraries are dual index** was selected.
    g. Experiment name, investigator name, description and date were entered.
    h. Read type – **paired end** – was selected.
    i. Cycles read: **301** was entered.
4. From the Home screen, Sequence was chosen to set up run.
5. From the Sequence Mode Selection screen, Local Run Manager was selected.
6. From the BaseSpace Options screen; use BaseSpace™ were selected. Then Next was selected.
7. The sample sheet was selected from the list of runs.
8. Next was selected to proceed to load the flow cell.

Clean the flow cell:

1. A new pair of powder free gloves were put on.
2. The flow cell was removed from the flow cell container.
3. The flow cell was rinsed with laboratory-grade water until both the glass and plastic cartridge were thoroughly rinsed of excess salts.
4. The flow cell was dried with lint-free lens tissue until it was completely dry.

Load the flow cell:

1. The flow cell compartment door was raised, and then the release button to the right of the flow cell clamp was pressed.
2. Holding onto the flow cells edges, it was placed on the flow cell stage.
3. The flow cell clamp was slowly pressed down to close it over the flow cell.
4. The flow cell compartment door was closed. Next was selected.

Load PR2 and Check the Waste Bottle:

1. The bottle of PR2 was removed from 2° to 8°C storage. It was inverted to mix and the lid was removed.
2. The reagent compartment door was opened.
3. The sipper handle was raised until it locked into place.
4. The wash bottle was removed and PR2 bottle was loaded.
5. The waste was emptied in an appropriate waste container.
6. The sipper handle was slowly lowered.

Load the Reagent Cartridge:

1. The reagent chiller door was opened. The reagent cartridge was inserted into the reagent chiller until the cartridge stopped.
2. The reagent chiller door was closed followed by the reagent compartment door. Next was selected. Start run was selected when the machine was ready.

Perform a Post-Run Wash:

1. When the run was completed, the Next button appears. Next was selected to perform a post-run wash.
2. A fresh solution with Tween 20 and laboratory-water was made:
    a. 5 ml 100% Tween 20 was added to 45 ml laboratory-grade water. These volumes resulted in 10% Tween 20.
    b. 25 ml 10% Tween 20 was added to 475 ml laboratory-grade water. These volumes resulted in a 0.5% Tween 20 wash solution.
    c. The solution was inverted five times to mix.
3. The wash component was prepared with a fresh wash solution:
    a. 6 ml of fresh wash solution was added to each reservoir of the wash tray.
    b. 350 ml was added to the wash bottle.
4. When the run was completed, start wash was selected.
5. The reagent compartment door and the reagent chiller door was opened and the reagent cartridge was replaced with the wash tray.
6. The PR2 bottle was replaced with wash bottle. The waste bottle was removed and its content discarded appropriately. It was then placed back to the reagent compartment.
7. The sipper handle was lowered and the compartment door was closed. Next was selected and the wash started.

4.6 Quality monitoring post sequencing

4.6.1 Quality control using MCS during sequencing

When sequencing using MCS, quality statistics to monitor parameters were provided during the run. Parameters such as **Cluster density**, **Cluster passing filter %**, **quality score (%Q30)** and **estimated yield** in megabases (Mb) were shown. During and after each run, these parameters were inspected. In table 5, specifications expected when using MiSeq V3 Reagent kit is given [69].

Table 5: *Specifications when using MiSeq V3 Reagent kit. Expected value and a description is given for each quality statistics.*

Quality statistics	Expected value	Description
Cluster density	1200-1400 K/ mm^2	Number of clusters per square millimeter on flow cell (K/ mm^2)
Cluster passing filter % (CPF%)	As high as possible	The percentage of cluster passing the Illumina chastity filter.
Quality score, %Q30	> 70% bases higher than Q30 at 2 × 300 bp	Average percentage of bases >Q30. Q30 = one base call in 1000 is predicted to be incorrect.
Output	13.2-15 Gb	The amount of output per flow cell.

4.6.2 Quality assessment using Sequence Analysis Viewer (SAV) post sequencing

When a run is finished, the output directory was opened in Sequence Analysis Viewer (SAV). Under the folder Charts, 1) **Flow cell chart**, 2) **Data By Cycle**, 3) **QScore Distribution**, 4) **Data By Lane** and 5) **QScore Heatmap** were shown. See figure 11 for an overview of the Chart folder.

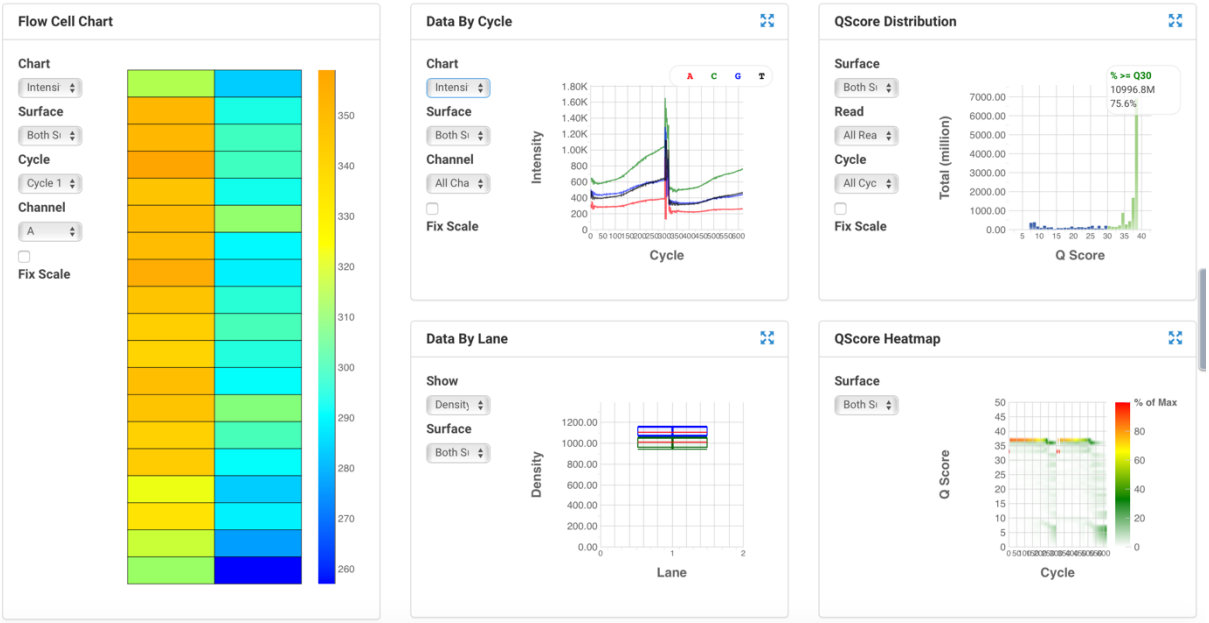

Figure 11: *Screenshot of a run from Sequence Analysis Viewer (SAV). Diagrams/ scales can be analyzed and viewed.*

1) The **Flow Cell Chart** shows color-coded graphical quality metrics per tile for the entire flow cell. This was used to judge local differences per cycle, per lane or per read in sequencing metrics on a flow cell.

2) The **Data By Cycle** plot shows the progression of quality metrics during a run as a line graph. This was used to judge the progression of quality metrics during a run on a cycle by cycle basis. Quality metrics used in this study: [70]

- **%Q30:** The percentage of bases with a quality score of 30 or higher. See figure 12.
- **%Base:** The percentage of clusters for which the selected base (A, C, T, or G) has been called.
- **Error rate:** The calculated error rate, as determined by a spiked in the PhiX control sample.

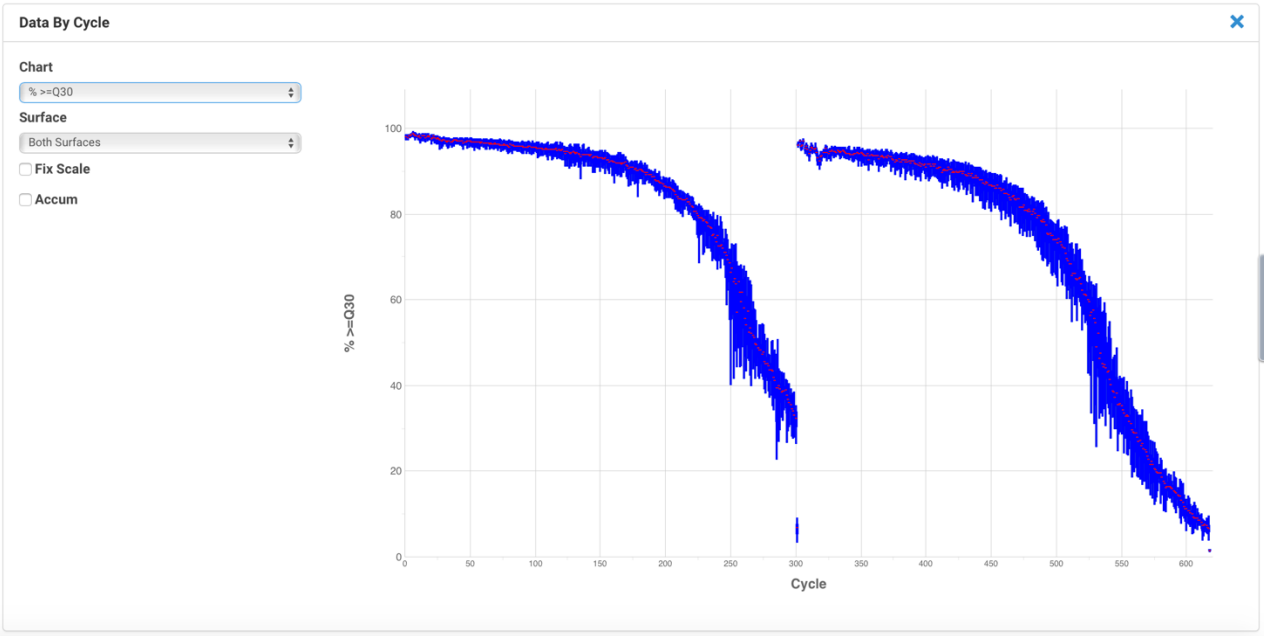

Figure 12: *Screenshot from a run in Sequence Analysis Viewer. This shows the Data By Cycle plot with chart %Q>=30.*

3) The **QScore Distribution** plot shows a bar graph that illustrates the number of bases by quality score. This was used to judge the QScore distribution for a run, which is an excellent indicator for run performance.
4) The **Data By Lane** plots shows quality metrics per lane. This was used to judge the difference in quality metrics between lanes.
5) **QScore Heatmap** is a heatmap of Q-scores. It shows a quick overview of the Q-scores over the cycles. This was not looked much into, except if the scores were very different than expected.
[70]

4.7 Bioinformatic analysis of sequence data

Raw data from the MiSeq run were stored on the MiSeq instrument under the folder output. This data consists of short-read sequences, sequence identifiers and quality scores stored in FASTQ format.

4.7.1 Quality check of raw data

FastQC v.0.11.7 [71] were used to evaluate the quality of the short-read sequences. FastQC provides a modular set of analyses to check if the data has any problems before doing further analysis. The program provides an output report for each sequenced isolate, showing negative or positive results within parameters such as base statistics, per base sequence quality, per sequences quality score, per base sequence content, per base GC content, per sequence GC content, per base N content, sequence length distribution, sequence duplication levels, overrepresented sequences and Kmer content. [72]

Another program that was used in addition to FastQC is MultiQC v1.4 [73]. MultiQC is a tool that aggregates all the gathered FastQC reports into one single report. This provides more overview of the samples, which makes it easier to analyze and sort out samples that are not within the optimal parameter ranges. [74]

Quality and adapter-based read trimming:
Trim Galore v0.6.1 [75], which uses CutAdapt and FastQC to apply quality and adapter trimming to FASTQ files, was used to trim the raw reads. It trims 1bp off each read at the 3'-end, trims low-quality bases (<Phred score 20) at the 3'-end, it removes any adapter sequences at the 3'-end and lastly removes read-pairs that are less than 20 bp long. Phred 20 indicates that 1/100 can be wrong.

4.7.2 Assembly

To reconstruct the genome from the raw data *de novo* assembly was performed.
Sequence reads were assembled into contigs, a stretch of continuous sequence generated by overlapping sequence reads [45]. See figure 13 for an illustration of the process. The coverage quality of *de novo* sequence data depends on the size and continuity of the contigs, and the sequencing depth [76].

De novo assembly was performed with Unicycler v0.4.4 [77], an assembly pipeline for bacterial genomes [78]. Unicycler uses SPAdes v3.13.0 [79], an assembly toolkit containing various assembly pipelines, and Pilon v1.22 [80] for assembly polishing.

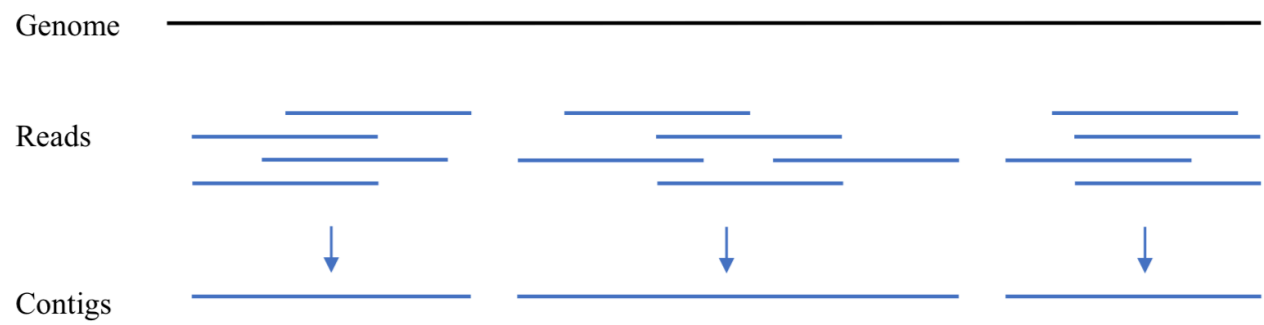

Figure 13: *De novo assembly. Overlapping short reads are aligned to make continuous sequences called contigs.*

4.7.3 Quality valuation of genome assembly

Quast v.4.6.3 [81] was used to evaluate the quality of the genome assembly. Several metrics are provided by Quast [82] (ideal quality):

- Number of contigs and length of contigs (<700 and long)
- Length of the largest contig (>0.135 Mbp)
- N50 – the length of the collection of all contigs of that length (or longer) that covers 50% of the assembly (minimum 30 000bp)
- L50 – number of contigs that are equal to or longer than N50 (as few as possible)
- GC% (57% expected for *K. pneumoniae*)

Quality check of sequencing depth:

The trimmed FastQ files were aligned against their respective assembled contigs with Seqdepth v1 (https://github.com/marithetland/SeqDepth), which uses the Burrows-Wheler Alignment v0.7.17-r1188 (BWA-MEM) [83] and Pickard Tools v2.17.8 [84] to create a Binary Alignment Map (BAM). It then uses SAMtools v1.7 [85] to calculate the overall sequence depth. SAMtools determines the depth of each position and average sequence depth is determined by dividing the average of all positions by the total genome size.

4.7.4 Phylogenetic analysis

RedDog v1beta.10.3 [86] is a comparative analysis pipeline that uses high-throughput sequences for large numbers of bacterial isolates [87]. To evaluate the relationship of the 722 isolates, the RedDog pipeline was used with the raw reads as input and the well characterized *Klebsiella pneumoniae* strain HS11286 (Genbank accession: NC_016845.1) as the reference genome.

Bowtie2 v2.2.5 [88], with the setting 'sensitive local-mapping', was used by RedDog to map the isolates' reads against the reference genome. SAMtools v1.7 [89] was used to identify single-nucleotide variants (SNVs) and FastTree created a maximum likelihood tree of the aligned isolates. To visualize the core genome SNV tree, msctrees-main.R (www.github.com/marithetland/msctrees/) was used.

4.7.5 Multi locus sequencing typing, species identification and resistance profiles

Kleborate v0.3.0 [90] is a software that identifies emerging pathogenic *Klebsiella pneumoniae* lineages, monitors antibiotic resistance and looks out for the convergence of antibiotic resistance and virulence. It screens *Klebisella* genome assemblies for multi-locus sequence types (STs), species and antibiotic resistance genes, including mutations leading to colistin or fluoroquinolone resistance. Kleborate uses Mash [91] to compare the assembly to a curated set of *Klebsiella* assemblies from NCBI (https://www.ncbi.nlm.nih.gov/assembly) and reports the species with the closest match. Kleborate was used to screen the sequenced *Klebisella pneumoniae* collection with the assembled contigs as input.

BLAST+ [92] was used to assess ESBL-encoding genes where Kleborate reported imprecise allele matches or incomplete coverage.

Kleborate determines MLST based on the *K. pneumoniae* MLST scheme hosted at institute Pasteur MLST system (https://bigsdb.pasteur.fr/klebsiella). Three isolates did not match any ST in that database and where therefore submitted to the institute Pasteur MLST curators and were assigned new ST types.

4.8 Antimicrobial susceptibility testing

From the population of 722 K. pneumoniae, a collection of 41 isolates were chosen for phenotypical characterization by antimicrobial susceptibility testing (AST). Broth microdilution was the method performed.

4.8.1 Micro broth dilution

Micro broth dilution (MBD) is a common method to determine the susceptibility of bacteria to antibiotics, both qualitatively and quantitatively. Sensititre™ Nephelometer, Sensititre AIM™ Automated Inoculation Delivery System, Sensititre™ Vizion™ Digital MIC Viewing System and Sensititre™ SWIN™ Software Epidemiology Module, all provided by Thermo Scientific, were used in this method. [57] A collection of 41 isolates were chosen from the population of 722 *Klebsiella pneumoniae* isolates. The selected isolates tested in this thesis consist mostly of ESBLs isolates and isolates with possible resistance against colistin.

Protocol:

1. Preparation of 0,5 McFarland inoculum suspension
    a. 3-5 colonies from an overnight culture on a non-selective blood agar were taken out using a sterile loop.
    b. The colonies were suspended in Sensititre™ destilled water and mixed. The density was measured to 0,5 McFarland using a nephelometer.
2. Preparation of Mueller-Hinton broth dilution
    a. 10µl of 0,5 McFarland suspension was added to 11ml of Sensititre™ Cation adjusted Mueller-Hinton broth w/ TES and mixed well.
3. Dispension of Mueller-Hinton suspension into the Sensititre NONAG4 and Sensititre NONAG5 plates using Sensititre automated inoculation delivery system (AIM)
    a. A Sensititre AIM was used to dispense 50µl inoculated broth into all the 96 wells on each of the micro titer plate containing different antimicrobial agents in different concentration.
    b. 1µl from the well with positive control was spread on to a blood agar plate. This colony count was done to see if the Mueller-Hinton suspension had a satisfactory concentration.
4. Incubation
    a. The inoculated plate was sealed and incubated for 18 hours at 35°C.

5. MIC-value determination using the Vizion plate reader.
    a. After the incubation, the positive control agar plates were inspected. 50-100 colonies were expected.
    b. The plates were read using the Vizion plate reader and Swin software. The MIC-values for each antimicrobial agent were set by marking the well with the lowest concentration that showed no growth. The software provided the MIC-values and resistance characteristics (susceptible, intermediate and resistant) according to EUCAST clinical breaking points.

5. Results

5.1 Raw data and assembly quality

The quality of the raw data output was analyzed with FastQC and the assembly quality with Quast. FastQC provided good quality scores for the majority of isolates, however, 20 isolates were re-sequenced due to poor quality score (see appendix B for details). After resequencing, all isolates (n=722) showed satisfactory qualities (table 6), see method section 4.7.3 for ideal qualities. GC% score ranged between 57-58.4%. Average sequence depth was 58.47.

Table 6: *Quality metrics for the 722 sequenced isolates.*

Quality metric	Average	Standard deviation	Range
Number of contigs [n]	114	51.5	36-331
Largest contig [Mbp]	0.58	0.24	0.13-1.9
Total length [Mbp]	5.4	0.17	4.9-6
GC score [%]	57.45	0.19	57-58.4
Sequencing depth [X]	58.47	18.39	16.39-115.6

N50 ranged from 34.9-982.1 kbp and L50 ranged from 2-45. The higher score of N50, the lower score of L50 (figure 14), i.e. the longer the contig, the fewer contigs. The majority of isolates laid within a range from L50=5-20 and N50=70-400 kbp.

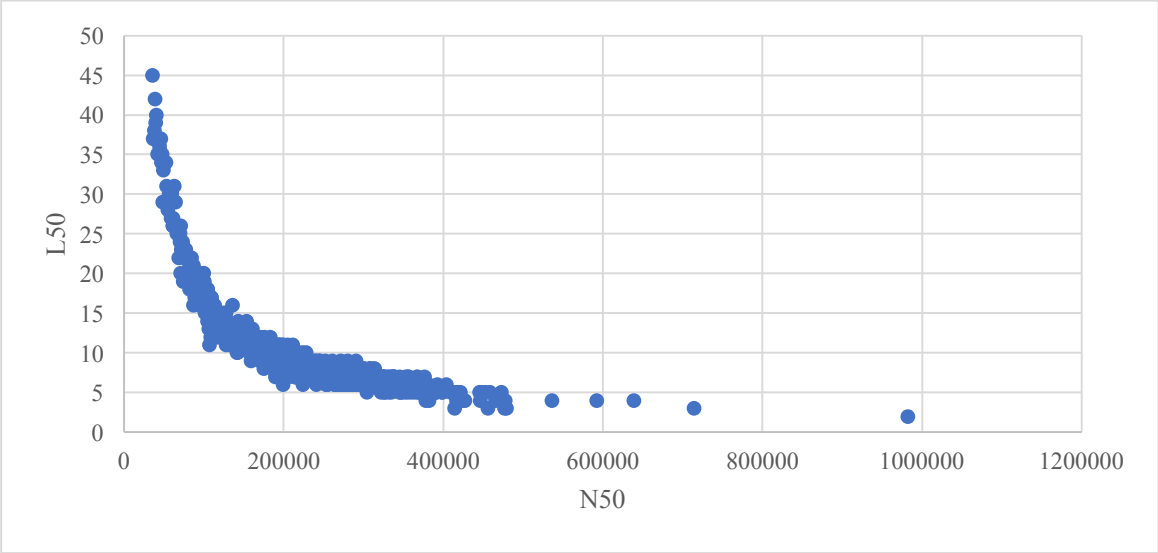

Figure 14: *The correlation between L50 and N50.*

5.2 Distribution of species, sequence types and ESBL-harboring isolates

5.2.1 Identification and distribution of *Klebsiella pneumoniae* species

Among the 722 *Klebsiella pneumoniae* isolates, four different species of *Klebsiella* were detected (table 7). *K. pneumoniae sensu stricto* was the most prevalent species (n=566), followed by *K. variicola* (n=120). 13 *K. variicola,* 2 *K. quasipneumoniae subs. quasipneumoniae* and 1 *K. pneumoniae sensu stricto* isolates showed a weak species match (see method section 4.7.5).

Table 7: *Distribution of species from the population of Klebsiella pneumoniae.*

Species	Proportion of strains	Number of strong match isolates	Number of weak match isolates
Klebsiella pneumoniae sensu stricto	78% (n=566)	565	1
Klebsiella variicola	17% (n=120)	107	13
Klebsiella quasipneumoniae subsp. similipneumoniae	3% (n=24)	24	-
Klebsiella quasipneumoniae subsp. quasipneumoniae	2% (n=12)	10	2
Total	100% (n=722)	706	16

5.2.2 Phylogenetic analysis of the sequenced *K. pneumoniae* population, multi locus sequence typing and prevalence of ESBL genes

A core chromosomal single-nucleotide variant (SNV) tree was made to visualize the phylogenetic relatedness of *K. pneumoniae* species. This tree (figure 15) divided *K. pneumoniae sensu stricto, K. variicola, K. quasipneumoniae subsp. similipneumoniae* and *K. quasipneumoniae subsp. quasipneumoniae* into different branches.

A high diversity of sequence types (STs) was discovered, with 378 different STs among the 722 isolates. STs occurring 1 or 2 times made up 60% of the isolates. In figure 15, STs that occur 5 or more times are highlighted using different colors. ST107 was detected as the most prevalent ST (n=67). Three new STs were detected and thus assigned 4009, 4010 and 4011 by the Institute Pasteur, who provides genotypic data for *K. pneumoniae* isolates based on MLST. The ESBL genes were more frequently detected in ST307 and ST45 than other STs.

A total of 50 $ESBL_A$-harboring isolates were detected in this population. One isolate containing an $ESBL_A$-gene was found among *K. quasipneumoniae subsp. similipneumoniae*, whereas all the other $ESBL_A$-genes were detected in *K. pneumoniae sensu stricto.*

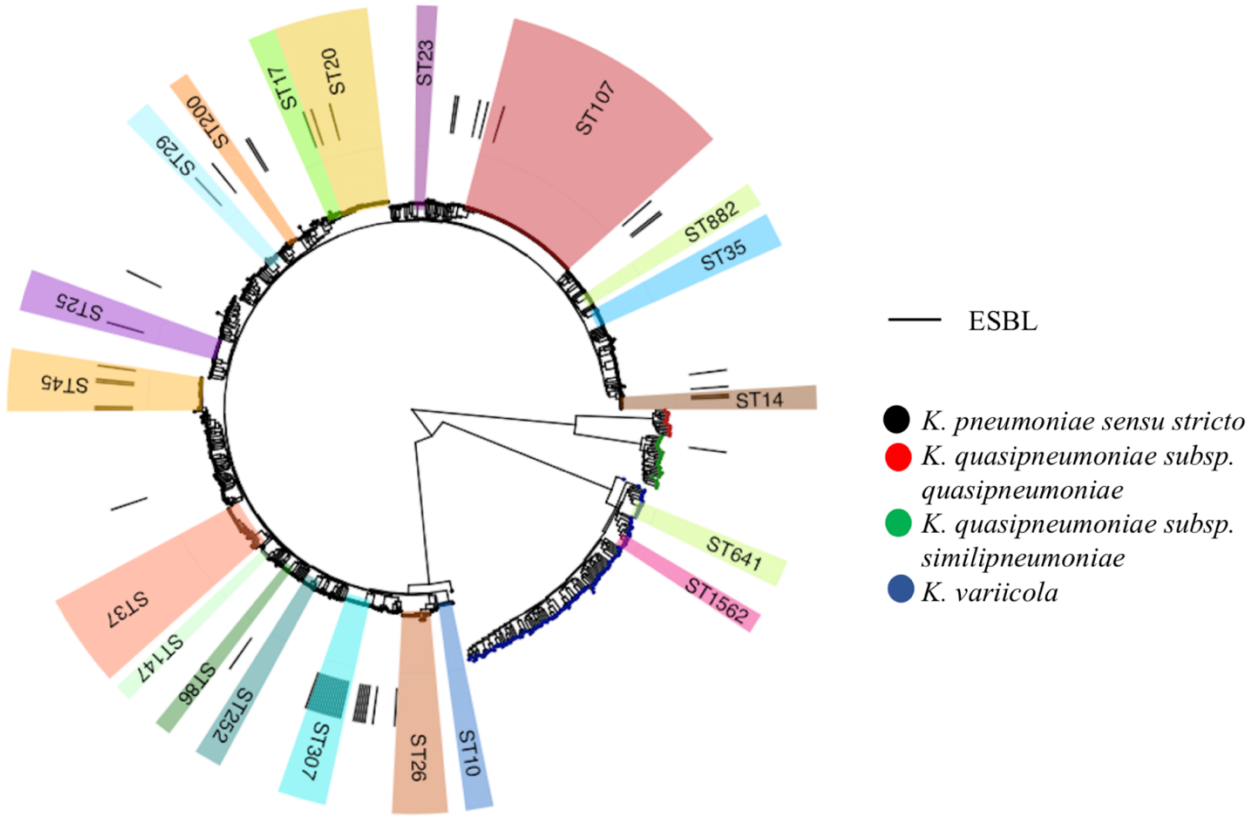

Figure 15: *A core chromosomal SNV tree highlighting species, ESBL and the most prevalent sequence types.*

5.2.3 Distribution of $ESBL_A$ genes in dominant $ESBL_A$-gene containing sequence types

STs that occurred 5 or more times with $ESBL_A$-genes are shown in table 8. ST307, which harbored $bla_{CTX\text{-}M\text{-}15}$, was found to be the most prevalent sequence type (n=11/50, 22%) among the $ESBL_A$-harboring isolates. All ST307, except one, were found with $bla_{CTX\text{-}M\text{-}15}$. Less than 1/3 of ST45 were found with an $ESBL_A$-gene.

Table 8: *Dominating ESBL-gene containing sequence type.*

Sequence type	ESBL encoding gene	Proportion of ST with ESBL gene	Proportion of all ESBL containing isolates
ST307	*bla*$_{CTX-M-15}$	11/12 (91%)	11/50 (22%)
ST307-1LV[1]	*bla*$_{CTX-M-15}$	2/2 (100%)	2/50 (4%)
ST45	*bla*$_{CTX-M-15}$	5/18 (28%)	5/50 (10%)

[1]ST307-1LV (6/7 loci match to ST307) included because it was highly similar to ST307

5.2.4 Geographical distribution of isolates with ESBL$_A$ encoding genes

Number of ESBL$_A$ isolates was compared to number of non-ESBL isolates from the different geographical regions (figure 16). In total, 50/722 (7%) isolates contained ESBL$_A$-genes. The number of ESBL$_A$-containing isolates in all regions ranged from 2-14 (average = 8.3, standard deviation = 4.9). The middle region showed the highest percentage (18%) of ESBL$_A$-harboring isolates compared to the other regions (6.5-7%).

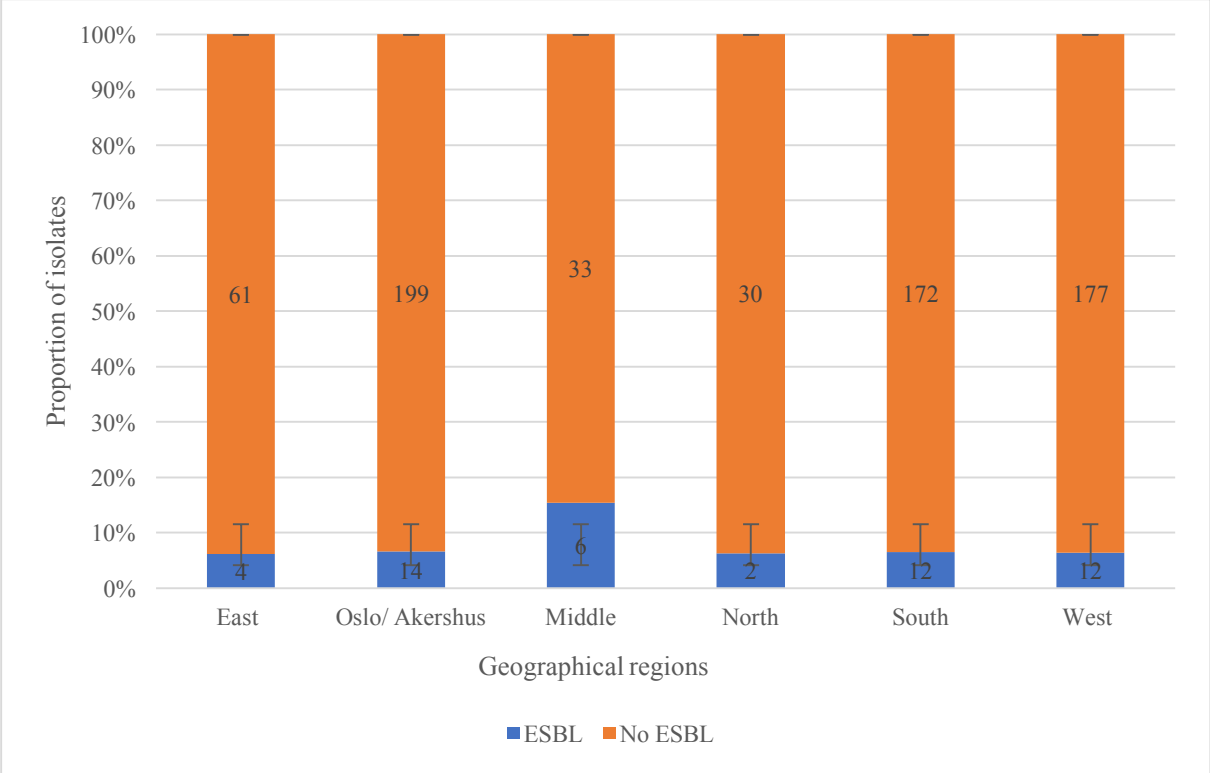

Figure 16. *Proportion of ESBL$_A$ isolates per region.*

5.3 Antimicrobial resistance – detection of genetic AMR determinants and phenotypical antimicrobial susceptibility testing

5.3.1a Detection of $ESBL_A$ encoding genes

Two families in $ESBL_A$ were detected, with 38 *bla*$_{CTX-M}$ and 14 *bla*$_{SHV}$ resistance genes. See table 9 for class A ESBL encoding genes. Four *bla*$_{CTX-M}$ were identified where *bla*$_{CTX-M-15}$ was the most prevalent (n=34/38, 89%). *bla*$_{CTX-M-15}$ was the most frequent in total (n=34/52, 65%). Seven *bla*$_{SHV}$ alleles were identified where *bla*$_{SHV-11}$ was the most prevalent (n=8/14, 57%). Two isolates were found with both a *bla*$_{SHV}$ and *bla*$_{CTX-M}$ allele. This included combinations of *bla*$_{SHV-11}$ and *bla*$_{CTX-M-15}$, and *bla*$_{SHV-1}$ and *bla*$_{CTX-M-15}$.

Table 9: *Class A ESBL encoding genes.*

Class A ESBL	$ESBL_A$ encoding genes
bla$_{CTX-M}$	*bla*$_{CTX-M-15}$ (n=34) *bla*$_{CTX-M-14}$ (n=2) *bla*$_{CTX-M-1}$ (n=1) *bla*$_{CTX-M-3}$ (n=1)
bla$_{SHV}$	*bla*$_{SHV-11}$ (n=8) *bla*$_{SHV-2a}$ (n=2) *bla*$_{SHV-40}$ (n=2) *bla*$_{SHV-1}$ (n=1) *bla*$_{SHV-2}$ (n=1)

5.3.1b Investigation of imprecise $ESBL_A$ encoding genes allele matches

Some $ESBL_A$ encoding genes were found with imprecise allele matches against the database. BLAST was used to investigate these $ESBL_A$ encoding genes (table 10).

Table 10: *$ESBL_A$ encoding genes that showed an imprecise match were investigated using BLAST.*

Kleborate ouput	BLAST results	Conclusion
CTX-M-15? (n=1)	Allele divided over two contigs	*$bla_{CTX\text{-}M\text{-}15}$*
SHV-2? (n=1)	100% coverage and 100% sequence identity to accession number[1]: MF4029301.1	*$bla_{SHV\text{-}2}$*
SHV-12*? (n=1)	100% coverage and 100% sequence identity to accession number[1]: MF402908.1	*$bla_{SHV\text{-}2a}$*
SHV-12* (n=1)	100% coverage and 100% sequence identity to accession number[1]: MF402908.1	*$bla_{SHV\text{-}2a}$*
SHV-13* (n=8)	100% coverage and 100% sequence identity to accession number[1]: CP032175.1	*$bla_{SHV\text{-}11}$*
SHV-13* (n=5)	1 SNV different from SHV-11, accession number[1]: CP032175.1	Reported as an ESBL – found as phenotypic susceptible. Excluded as an ESBL gene[2]
SHV-13* (n=2)	3 SNV different from SHV-11, accession number[1]: NG_062297.1	Reported as an ESBL, no phenotypic results. Excluded as an ESBL gene[2]
SHV-101* (n=7)	1 SNV different from SHV-101 and 1 SNV different from SHV-1	Reported as an ESBL – found as phenotypic susceptible. Excluded as an ESBL gene[2]
SHV-101* (n=1)	100% coverage and 100% sequence identity to accession number[1]: CP014123.1	*$bla_{SHV\text{-}1}$*

[1]GenBank accession number

[2]See appendix C for details

5.3.2a Distribution of resistance determinants against important antimicrobial drug classes

148/722 (20.5%) isolates were detected with genotypic resistance determinants against the investigated classes of antibiotics, β-lactam, aminoglycosides, fluoroquinolones, sulfamethoxazole, trimethoprim and colistin. Isolates contained up to 5 different resistance determinants against each class of antibiotics (table 11).

Table 11: *Number of different resistance determinants found in isolates (n=722).*

Class of antibiotics	Isolates containing number of resistance determinants:					
	0	1	2	3	4	5
β-lactam (extended-spectrum)	672 (93%)	48 (6.6%)	2 (0.4%)	-	-	-
Aminoglycosides	623 (86.3%)	21 (2.9%)	39 (5.4%)	29 (4%)	7 (1%)	3 (0,4%)
Fluoroquinolones	654 (90.6%)	30 (4.2%)	-	15 (2%)	2 (0.3%)	-
Fluoroquinolones SNV[1]		13 (1.8%)	6 (0.8%)	2 (0.3%)	-	-
Sulfamethoxazole	622 (86.1%)	76 (10.5%)	21 (3%)	3 (0.4%)	-	-
Trimethoprim	626 (86.7%)	95 (13.2%)	1 (0.1%)	-	-	-
Trimethoprim/ sulfamethoxazole[2]	615 (85.2%)	17 (2.3%)	64 (8.9%)	22 (3%)	4 (0.5%)	-
Colistin deletion[3]	717 (99.3%)	5[1] (0.7%)	-	-	-	-

[1]Mutiations in chromosomal gene GyrA or ParC conferring resistance

[2]Drug combination used in clinical practice, for comparison to AST

[3]Truncations in chromosomal gene MgrB or PmrB conferring resistance

5.3.2b Resistance determinant gene variants

A wide selection of resistance determinant variants was found (table 12).

Table 12: *Resistance determinant variants*.

Class of antibiotic	Number of isolates with resistance determinants	Gene variants
β-lactam (extended-spectrum)	50	*bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-3}}$, *bla*$_{\text{SHV-1}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{SHV-2}}$, *bla*$_{\text{SHV-2a}}$, *bla*$_{\text{SHV-40}}$
Aminoglycosides	13	Aac3-IIa, AadA2, AadA5, Aph3-Ia, Aph4-Ia, ArmA, Sat-2A, StrA, StrB,
Aminoglycosides – imprecise allele match	67	Aac3-IIa*[1], Aac3-IId*[1], AadA16*[1], AadA2*[1], AadA4*[1], Aph3-Ia*[1], StrA*[1], StrB*[1],
Aminoglycoside – partial match	1	Aac3-Iva?[2], AadA1-pm?[2], StrB?[2]
Aminoglycosides – partial match and imprecise allele match	18	Aph3-Ia*?[1,2], Aph3"Ia*?[1,2], StrA*?[1,2], StrB*?[1,2], Aac3-Iva*?[1,2], Aac3-Iva*?[1,2], AadA1-pm*?[1,2]
Fluoroquinolones	42	GyrA-83A, GyrA-83F, GyrA-83Y, GyrA-87G, ParC-80R, ParC-80l, GyrA-87N, GyrA-87Y, Qnr-S1, GyrA-83l, QnrB2, QnrB4,
Fluoroquinolones – partial match	25	QnrB1?[2]
Fluoroquinolones – imprecise allele match	1	QnrB5*[1]
Sulfamethoxazole	78	SulI, SulII, SulIII
Sulfamethoxazole – imprecise allele match	10	SulI?[2]
Sulfamethoxazole – partial match	12	SulII*[1], SulI*[1]
Trimethoprim	83	DfrA1, DfrA12, DfrA14, DfrA17, DfrA25, DfrA27, DfrA5, DfrA7
Trimethoprim – partial hits	13	DfrA1*[1], DfrA14*[1], DfrA15b*[1], DfrA5*[1]
Colistin[3]	5	MgrB-53%, MgrB-89% (end of contig), PmrB-65%, MgrB-57%

[1]* Indicates imprecise allele matches

[2]? Indicates that the length of match is less than the length of the reported allele

[3]Truncations conferring resistance

5.3.3 Detection of multidrug resistance

In total, 46/722 (6.4%) isolates were genotypic multidrug resistant, containing resistance determinants against 3 or more classes of antibiotics. See figure 17 for the proportion of multidrug resistant isolates per class of antibiotic. Trimethoprim and sulfamethoxazole are counted as one drug class due to drug combination used in clinical practice, for comparison to AST.

31/35 (88%) of the $ESBL_A$-containing multidrug resistant isolates harbored $bla_{CTX\text{-}M\text{-}15}$. $bla_{CTX\text{-}M\text{-}15}$ co-existed with aminoglycosides, fluoroquinolones, trimethoprim/ sulfamethoxazole resistance determinants in 30 of these isolates. One isolate which harbored $bla_{CTX\text{-}M\text{-}15}$, also harbored determinants conferring resistance to aminoglycosides, fluoroquinolones and colistin. A truncation of MgrB-89%, was the resistance mechanisms found in the multidrug resistant isolate concerning colistin. 43/46 (93%) of the multidrug isolates had a combination of aminoglycosides, fluoroquinolones trimethoprim/ sulfamethoxazole resistance determinants.

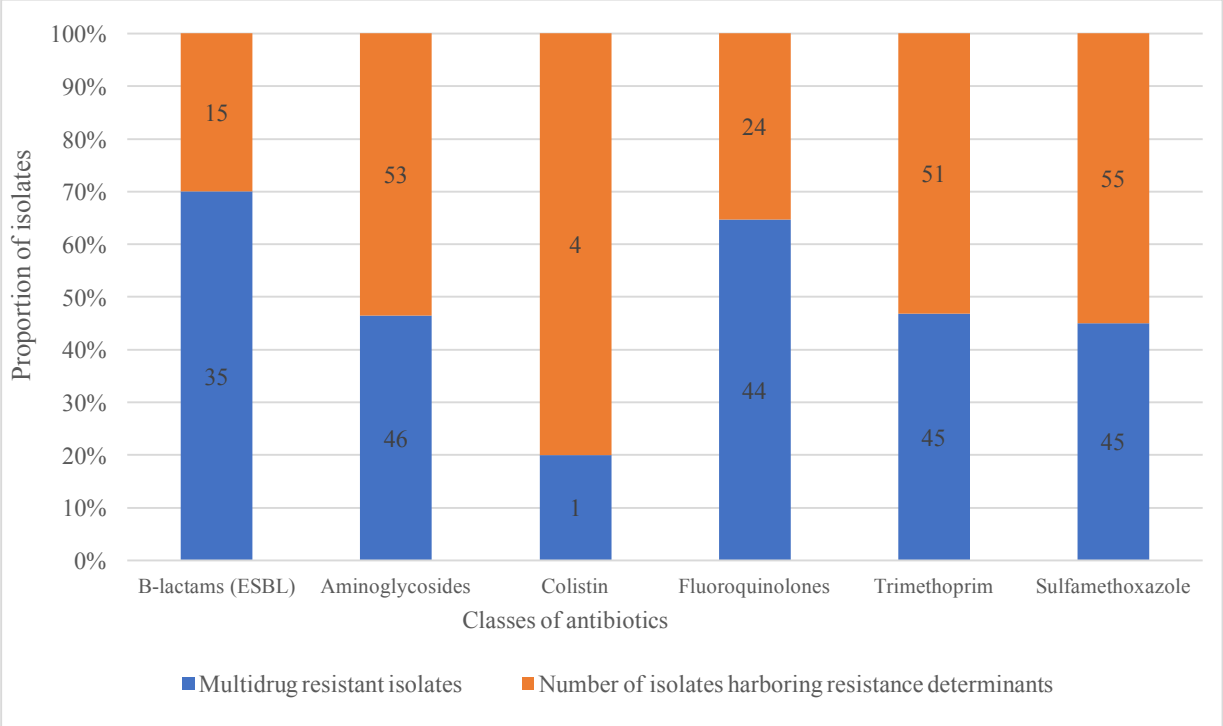

Figure 17: *Proportion of multidrug isolates in the different classes of antibiotics. Blue color indicated multidrug isolates for the specific class. Orange color indicated isolates that harbored resistance determinants against the specific class, but were not multidrug resistant.*

STs that occurred 3 or more times among the multidrug resistant isolates are presented in table 13. ST307 followed by ST25 were the most prevalent STs among the multidrug resistant isolates. ST307, ST307-1LV and ST45 were found with *bla*$_{\text{CTX-M-15}}$. ST147 and ST25 was detected with determinants encoding resistance to aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazole. ST101 was found with a truncation conferring resistance to colistin and determinants encoding resistance to β-lactam, aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazole.

Table 13: *Sequence types frequently detected in multidrug resistant isolates.*

Sequence type	Proportion of ST found among MDR isolates
ST307	11/12 (91%)
ST307-1LV[1]	2/2 (100%)
ST45	5/18 (27.7%)
ST147	4/5 (80%)
ST25	3/12 (25%)
ST101[2]	1/3 (33%)

[1]ST307-1LV (6/7 loci match to ST307) included because it was highly similar to ST307

[2]ST101 included because one isolate was MDR with colistin truncation MgrB-89%

5.3.4 Comparison of phenotypic antimicrobial susceptibility testing with detection of resistance determinants

Phenotype determined with antimicrobial susceptibility testing (AST) using micro broth dilution (MBD) was compared to genotypic resistance determinants found as shown previously in table 12. MBD were performed on 41 isolates, where the selection consisted mostly of ESBL isolates (n= 37/41, 90%). Details of all micro broth dilution results can be found in appendix D, included the MIC-values (appendix D.6).

5.3.4.1 β-lactams

The results for β-lactams are presented in table 14. 39/41 (95%) isolates matched with both genotypic and phenotypic results, whereas one was detected with partial match. One strain was found to be phenotypic resistant to ceftazidime and cefotaxime where no β-lactam resistance gene was detected. See appendix D.1 for details.

Table 14: *Comparison of micro broth dilution and β-lactams resistance genes.*

Micro broth dilution		Resistance genes combinations	Number of strain(s)	Comparison
Ceftazidime	Cefotaxime			
R	R	*bla*$_{\text{CTX-M-15}}$	31	Match
R	R	*bla*$_{\text{CTX-M-14}}$	2	Match
R	R	*bla*$_{\text{CTX-M-3}}$	1	Match
R	R	*bla*$_{\text{SHV-1}}$;*bla*$_{\text{CTX-M-15}}$	1	Match
R	R	*bla*$_{\text{SHV-11}}$;*bla*$_{\text{CTX-M-15}}$	1	Match
R	R	-	1	No match
I	R	*bla*$_{\text{CTX-M-1}}$	1	Partial match
S	S	-	3	Match

5.3.4.2 Aminoglycosides

See table 15 for phenotypic results from micro broth dilution compared to genotypic resistance regarding aminoglycosides. 36/41 (87%) isolates matched with both genotypic and phenotypic results. One strain was found to be phenotypic resistant to aminoglycosides, although no aminoglycoside resistance gene was detected. See appendix D.2 for details.

Table 15: *Comparison of micro broth dilution and aminoglycoside resistance genes.*

Micro broth dilution			Resistance genes combinations	Number of strain(s)	Comparison of phenotypic and genotypic results
Amikacin	Gentamicin	Tobramycin			
S	S	S	StrB;StrA*[1]	7	Match
S	S	S	-	8	Match
S	S	S	AadA2*[1];StrB*[1];StrA*[1];Aph3-Ia*[1]	2	Match
S	R	R	StrB;StrA*[1];Aac3-IIa*[1]	15	Match[3]
S	R	R	StrB*[1];StrA*[1];AadA2*[1];Aac3-IIa*[1]	1	Match[3]
S	R	R	StrB*?[1,2];StrA*[1];Aac3-IId*[1]	2	Match[3]
S	R	R	Aac3-IIa*[1]	1	Match[3]
I	S	R	StrB;StrA*[1]	4	No match
R	R	R	-	1	No match

[1]* Indicates imprecise allele matches

[2]? Indicates that the length of match is less than the length of the reported allele

[3]Indicated that the resistance gene was present

5.3.4.3 Fluoroquinolones

Table 16 represents the results from micro broth dilution and resistance determinants combinations regarding fluoroquinolones. 39/41 (95%) isolates matched with both genotypic and phenotypic resistance. One isolate showed phenotypic resistance, but no fluoroquinolone resistance determinants was detected. See appendix D.3 for details.

Table 16: *Comparison of micro broth dilution and fluoroquinolones resistance determinants.*

Micro broth dilution Ciprofloxacin	Resistance determinants combinations	Number of strain(s)	Comparison of phenotypic and genotypic results
R	QnrB1?[1]	13	Match[2]
R	GyrA-83I;ParC-80I	2	Match
R	QnrB1?[1];GyrA-83I;ParC-80I	10	Match[2]
R	QnrS1	7	Match
R	GyrA-83Y;GyrA-87N;ParC-80I	1	Match
R	-	1	No match
S	QnrB1?[1]	1	No match[2]
S	-	6	Match

[1]? Indicates that the length of match is less than the length of the reported allele

[2]Indicated that the resistance gene was present

5.3.4.4 Trimethoprim/ sulfamethoxazole

The results from micro broth dilution and resistance genes combinations regarding trimethoprim/ sulfamethoxazole are presented in table 17. 38/41 (93%) isolates matched with both genotypic and phenotypic resistance, whereas 3/41 (7%) had partial match with gene encoding resistance to one drug class present. Two were found with phenotypic resistance and resistance gene detected was DfrA14, and one were found to be phenotypic susceptible and resistance gene detected was SulII. See appendix D.4 for details.

Table 17: *Comparison of micro broth dilution and trimethoprim/sulfamethoxazole resistance genes. Green color indicates match, grey color indicates partial match.*

Micro broth dilution Trimethoprim/ Sulfamethoxazole	Resistance genes combinations	Number of strain(s)	Comparison of phenotypic and genotypic results
R	SulII, DfrA14	26	Match
R	DfrA14	2	Partial match
R	SulII;SulI, DfrA12	3	Match
R	SulII*[1], DfrA14	2	Match
S	SulII	1	Partial match
S	-	7	Match

[1]Indicates imprecise allele match

5.3.4.5 Colistin

For colistin, 5 isolates were found with genotypic resistance determinants (truncations of MgrB or PmrB). In table 18, results from micro broth dilution and resistance determinants combinations are presented. 38/41 (93%) isolates matched with phenotypic and genotypic results. 38 of the isolates were susceptible to colistin, where two isolates were found with resistance determinants. 3 isolates were resistant, where two isolates were found with resistance determinants. See appendix D.5 for details.

Table 18: *Results from micro broth dilution and resistance determinants combinations regarding colistin. For MBD: S = susceptible, R = resistant, NA = not analyzed.*

Isolates	Micro broth dilution	Resistance determinants	Comparison of phenotypic and genotypic results
NK-7	S	PmrB-65%	No match
NK-15	S	MgrB-53%	No match
NK-23	R	MgrB-89%	Match
NK-36	R	PmrB-65%	Match
NK-3	R	-	No match
NK-42	NA	MgrB-57%	NA in MBD

6. Discussion

6.1 Discussion methods

6.1.1 Next-generation Sequencing

One of the challenges faced during sequencing, was the occurrence of over clustering. Which means that there is too much DNA library fragments for the flow cell to handle. This resulted in reduced quality scores. Optimal cluster density is found in the range 1200-1400 K/ mm^2, higher values are considered over clustering.

Over clustering may be due to high concentration of input genomic DNA before Tagmentation. However, all samples were normalized to 0,2 ng/µl and should hereby not lead to over clustering.

Another suspecting cause leading to over clustering, was the contamination of primer adapters. One explanation could be that the AMPure XP beads did not work optimally, which led to the primer adapters being sequenced rather than the DNA library. It was examined for primer adapters using Bioanalyzer, where some primer adapters were detected. This could indicate contamination in the AMPure XP beads and therefore a new bottle was used. No more primer adapters were detected in Bioanalyzer for the remaining isolates for sequencing.

Despite this effort to reduce over clustering, it did not always work out. The load onto the flow cell was decreased as a last attempt to avoid over clustering. This gave satisfactory results regarding Q30 score, cluster density and cluster passing filter and was perfectly good to use for analysis.

6.1.2 Antimicrobial susceptibility testing

Some of the isolates from micro broth dilution were found with conflicting outcomes.
This could indicate a weakness in the procedure which can be explained by low or high concentration of bacterial inoculum affecting the growth of bacteria. And, poor mixing could make the McFarland inoculum suspension not homogenous which could lead to different inoculated concentration of bacteria. Some strains were sticky and difficult to dissolve. This can be solved by mixing until the solution is completely homogenous.

6.1.3 Bioinformatics analysis

Due to over clustering, some isolates showed poor quality scores in FastQC and in Quast, hereof needed to be re-sequenced.

Kleborate was used in this thesis for species identification, MLST and detection of resistance determinants. Kleborate screens for resistance determinants against the ARG-Annot database of acquired resistance determinants (SRST2 version). Imprecise match is indicated with ‘’*’’, and ‘’?’’ indicates that the length of the allele is less than the length of the reported allele. Many resistance determinants in this population reported ‘’*’’ or ‘’?’’. An explanation for ‘’?’’ could be poor coverage in the actual part of the sequence. This could be solved by re-sequencing. Imprecise match may be explained by a missing part of the determinant leading to SNV differences and incomplete match. This database is very good for providing an overview of gene-content. One weakness is that there can be alleles not registered in the ARG-Annot database, which results in uncomplete match. For the ESBLs, BLAST+ was used to assess the ESBL encoding genes reported with imprecise allele match or incomplete coverage. For the other classes of antibiotics, there was not enough time to check all the imprecise alleles with BLAST+. This is something to be done for further investigations.

Kleborate provides an identification of species where a weak match could indicate a novel linage or hybrid. For the isolates reporting weak match, further phylogenetic analysis could be done.

Screening this population of 722 *K. pneumoniae* isolates, for resistance determinants, MLST and identification of species, with other methods than NGS and computational analysis would be very time consuming and very expensive.

6.2 Discussion results

6.2.1 Raw data and assembly quality

The quality metrics for the 722 *K. pneumoniae* isolates showed all satisfactory results. It is desirable that number of contigs are fewer than 700, and they were found in the range 36-331. For largest contig, good quality is indicated within the detected range: 0.13-1.9 Mbp. The average of the total length was found to be 5.4 Mbp and in the range 4,9-6 Mbp. This indicated satisfactory results where the genome of *K. pneumoniae* = 5,3 Mbp. [93] GC% score ranged between 57-58%, as expected for *K. pneumoniae.* [94] N50 and L50 and score showed satisfactory results, where L50 should be as low as possible and N50 as high as possible, where a minimum should be 30 kbp.

The sequencing depth was found in the range 16.39-115.6 and with average value of 58.47. The standard deviation was found to be 18.39, meaning that 95% of the isolates lies within the range of 22.4-94.5. This indicates good quality for the majority of isolates, where coverage should be as high as possible. A good indication for lower limit of sequencing depth in *K. pneumoniae* can be 30. [95] For the isolates detected with low coverage, questions whether the quality is good enough or not arises. Other quality metrics, such as GC% score, largest contig, number of contigs and total length, were checked for isolates with low coverage, and isolates were therefore included based on other satisfactory qualities.

6.2.2 Distribution of species and phylogenetic analysis

Four species were detected in this population, where *K. pneumoniae sensu stricto* was found to be the most prevalent strain. This shows that *K. pneumoniae sensu stricto* is the most observed strain in an unselected collection of invasive isolates from adult humans. The core-chromosomal SNV tree displays how phylogenetically close related the four species are, where the phylogroups can be reliably identified based on genome sequencing. [96] The results showed some weak species matches, which may indicate that these were a novel linage or a hybrid. [90] This could have been further analyzed to confirm whether it’s a hybrid or novel linage.

6.2.3 Multi locus sequence typing, $ESBL_A$-harboring sequence types and geographic distribution of $ESBL_A$-genes

378 different sequence types were detected, showing a high diversity. ST107 was found to be the most prevalent sequence type in this population. However, no resistance genes of significance were found with this ST except for one isolate, where $bla_{CTX\text{-}M\text{-}1}$ were found. By being the most prevalent in this population could indicate that this ST is evolving and spreading over time. However, why there is a high prevalence of ST107 in this population is unknown. Further phylogenetic analysis could be done to closer investigate ST107.

11/12 ST307 and 2/2 ST307-1LV were found with $ESBL_A$ encoding gene $bla_{CTX\text{-}M\text{-}15}$. This is consistent with research linking ST307 to production of CTX-M-15. [97] One possible explanation for the one ST307 missing $bla_{CTX\text{-}M\text{-}15}$, could be that the plasmid was lost. This isolate could also be re-sequenced and tested in AST to confirm whether the resistance gene is present.

5/18 ST45 carried $bla_{CTX\text{-}M\text{-}15}$. Recent studies link ST45 to the production of $bla_{CTX\text{-}M\text{-}15}$, which indicates an emerge of a new $ESBL_A$-producing *K. pneumoniae* ST. [98]

The middle region was found with highest percentage of $ESBL_A$-encoding genes (18%), but only with 6 $ESBL_A$-harboring isolates. However, the high percentage can be explained by the low numbers of isolates from this region. The other regions were located within the standard deviation, where about 6.5-7% of isolates harbored $ESBL_A$-genes. Oslo/Akershus was detected with highest number of $ESBL_A$-harboring isolates, but with a low percentage. According to NORM (Norwegian Surveillance System of Antibiotic Resistance in Microbes), the percentage of ESBL isolates found in the different locations varies over time. In NORM-VET 2017, the south region was detected with highest percentage of ESBL resistance genes, followed by north, Oslo/Akershus, west, middle and east region, in this order. [99] Due to this variation from year to year, geographic excretion cannot be verified. Our data is however not quite representative compared to NORM, due to a fewer hospitals included in this population. According to NORM, the proportion of ESBL resistance have increased since 2002, and would probably increase even more in the years to come. [99]

6.2.4 Prevalence of known AMR-determinants

50 $ESBL_A$-harboring isolates were observed in this population. $bla_{CTX\text{-}M\text{-}15}$ (n=34) was the dominate $ESBL_A$-gene followed by $bla_{SHV\text{-}11}$ (n=8). $bla_{CTX\text{-}M\text{-}15}$ is now the dominant ESBL type, where it is the main enzyme currently encountered in *K. pneumoniae*. [15] This is consistent with the current antimicrobial resistance situation where the SHV and TEM family have been replaced by the CTX-M family where it became the dominant ESBL in most parts of the world, including Norway. [38]

In total, 20.5% of the *K. pneumoniae* isolates in this population had resistance genes encoding at least one or more drug groups of interests. In line with NORM surveillance, this shows less resistance compared to the EARS-NET report from 2015, where 1/3 of the *K. pneumoniae* isolates were resistant to at least one of the antibiotics groups (fluoroquinolones, third-generation cephalosporins, aminoglycosides and carbapenems). [11] This indicates that Norway (this population) have lower resistance percentage compared to the rest of Europe. This is also consistent with the EARS-NET report where lower resistance percentages are reported in the northern European countries compared to the southern and south-eastern parts of Europe. Misuse and overuse accelerate the process for bacteria becoming resistant. [1] Lower resistance percentage in Norway may be a consequence of more restrictions and guidelines regarding the use of antibiotics. [100]

In this population, five isolates were detected with determinants encoding resistance to colistin. The increase in resistance against colistin makes infections caused by carbapenem and multidrug resistant *K. pneumoniae* difficult to treat. [3]

There is an overall high detection of determinants encoding resistance to aminoglycosides, fluoroquinolones, trimethoprim and sulfamethoxazole, where 99, 68, 100 and 96 isolates contained resistance determinants respectively. Indicating that these widely-used groups of antibiotics may be less effective due to this detection of resistance determinants.

6.2.5 Genetic characteristics of multidrug resistant isolates

A total of 6.4% of the *K. pneumoniae* isolates were found to be multidrug resistant. This percentage is much lower than reported in other research, which is also consist with the EARS-NET report as previously mentioned, where the northern countries have a lower resistance percentage. [101] However, Norway have shown an increased detection of multidrug resistant isolates since 2017 according to the EARS-NET report. [40]

Out of the 46 MDR isolates, 76% (n=35) of these were found with an $ESBL_A$-gene. Indicating a correlation between $ESBL_A$-genes and multidrug resistant bacteria.

93% of the multidrug isolates had the combination of aminoglycoside, fluoroquinolones and trimethoprim/ sulfamethoxazole resistance determinants. This may indicate a connection between these classes and the emergence of multidrug resistant *K. pneumoniae*. A troubling finding like this makes infections caused by *K. pneumoniae* harder to treat with these antibiotics.

70% of the $ESBL_A$-harboring isolates were found to be multidrug resistant. 31 (88%) of the $ESBL_A$-harboring multidrug resistant isolates was found with $bla_{CTX\text{-}M\text{-}15}$ and co-existed with aminoglycosides, fluoroquinolones, trimethoprim/ sulfamethoxazole resistance determinants. This can be explained by plasmids responsible for ESBL production often carry other genes encoding resistance to other drug classes. Hereby making the antibiotic options for treatment of MDR bacteria extremely limited. [33]

1/5 of colistin isolates were multidrug resistant, where MgrB-89% co-existed with determinants encoding resistance to aminoglycoside, fluoroquinolones and β-lactams. Several studies have reported the emergence of colistin resistance in MDR *K. pneumoniae* arising from loss-of-function mutations of the MgrB gene. [10] This deletion was found at the end of one contig, where a truncation of the gene <90% confers resistance. However, this isolate shows both phenotypic and genotypic resistance to colistin indicating that this isolate is resistant to colistin. This isolate was found with ST101. In the dissemination of colistin resistance and increased mortality of *K. pneumoniae* infection, ST101 may become a global threat. [102]

The STs associated with the MDR isolates in this population were predominantly ST307, ST45, ST147 and ST25. ST307 was detected 11/12 times with *bla*$_{\text{CTX-M-15}}$ among the MDR isolates. ST45 was found 5/18 times among the MDR isolates, where *bla*$_{\text{CTX-M-15}}$ was detected. As previously described, ST307 is linked to the production of *bla*$_{\text{CTX-M-15}}$ and ST45 may be linked to production of *bla*$_{\text{CTX-M-15}}$. Plasmids responsible for ESBL production frequently carry other determinants encoding resistance to other classes of antibiotics. Indicating that ST307 is linked to production of *bla*$_{\text{CTX-M-15}}$ and multidrug resistance. And may indicate an emergence of ST45 among MDR isolates.

ST147 was detected 4/5 times among the MDR isolates with determinants encoding resistance to aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazole. This may indicate that this ST is associated with MDR. However, recent studies have reported ST147 frequently associated with NDM-1 and OXA48-like carbapenemases among Indian isolates. [103]

ST25 was found 3/12 times among the MDR isolates with respectively determinants encoding resistance to aminoglycosides, fluoroquinolones and trimethoprim/ sulfamethoxazole. This ST is associated with septicaemia in pigs and emerged in England in 2011 and observed since then. [104] However, it is well known that antimicrobial resistant bacteria can be spread between animals and humans. [3] Based on the few ST25 found among the MDR isolates, the possibility of associating ST25 to MDR is not very likely.

6.2.6 Comparison of phenotypic antimicrobial susceptibility testing with detection of resistance determinants

95% of the isolates tested in micro broth dilution regarding β-lactams matched with both phenotypic and genotypic resistance or susceptibility. 36 isolates were found to be resistant to both ceftazidime and cefotaxime, where a *bla*$_{\text{CTX-M}}$ gene was present. One isolate was found to be susceptible with increased exposure to ceftazidime and resistant to cefotaxime, where *bla*$_{\text{CTX-M-1}}$ was detected. The high rate of resistance to third-generation cephalorsporins, ceftazidime and cefotaxime, can be explained by the high prevalence of *bla*$_{\text{CTX-M-15}}$ and other alleles from the *bla*$_{\text{CTX-M}}$ family.

87% of isolates regarding aminoglycosides matched with phenotypic and genotypic results. 19 matching isolates were found to be susceptible to amikacin and resistant to both gentamicin and tobramycin. Aac3-IIa* and Aac3-IId* explains the resistance detected in gentamicin and tobramycin. [105] Indicating that these genes encode resistance despite being detected with imprecise match. StrA and StrB does not confer resistance to amikacin, gentamicin and tobramycin. [106] However, 4 isolates were found to be intermediate, susceptible and resistant to these drugs respectively, where StrA and StrB resistance genes was detected. This can be explained by too high concentration of bacteria in the suspension which is causing too much growth.

Regarding fluoroquinolones, 95% of isolates matched with phenotypic and genotypic results. QnrB1, QnrS1 and mutations conferring resistance, GyrA and ParC, encodes resistance to ciprofloxacin. 13 isolates with QnrB1? showed phenotypic resistance, indicating that the determinant confers resistance despite having incomplete coverage. Research link QnrB1 to resistance against fluoroquinolones. [107] One isolate with QnrB1? was found to be susceptible, further indicating that the protein conferring resistance may not be present.

93% of the isolates regarding trimethoprim/sulfamethoxazole matched with both genotypic and phenotypic resistance, whereas 3/41 (7%) had partial match with gene encoding resistance to one drug class present. Two isolates with partial match, where only DfrA was detected, was found to be resistant. One isolate was found to be susceptible with SulII resistance gene present. This may indicate that DfrA confers resistance to both classes, while SulII only confers resistance to sulfamethoxazole. Or it can be explained by poor mixing and low concentration of bacteria in the suspension.

Regarding colistin, 97.6% of isolates matched with phenotypic and genotypic results. Three isolates were found to be phenotypic resistant to colistin, and in two of these isolates genotypic resistance determinants MgrB-89% and PmrB-65% were detected. Resistance to colistin is explained by truncations in the MgrB and PmrB gene. Kleborate determined <90% as a truncation conferring resistance. One isolate was found to be phenotypic resistant but no genotypic resistant determinant was present. Two isolates found to be phenotypic susceptible were detected with truncations PmrB-65% and MgrB-53%. Since no phenotypic resistance was detected, low concentration of bacteria in the suspension could be a reason for no or too little growth.

6.2.6.1 Conflicting results

One isolate is found to be phenotypic resistant to aminoglycosides, β-lactams and fluoroquinolones, but have no genotypic determinant encoding resistance to these classes. This isolate was found with phenotypic and genotypic resistance to colistin, truncation PmrB-65%. Which indicates that resistance to colistin could lead to phenotypic resistance in other classes of antibiotics. It can also be explained by too high concentration of bacteria leading to growth in all types of antibiotics. Further analysis to ensure whether it causes resistance to other classes of antibiotics, is to investigate this isolate once more in AST.

N-7 was found to be phenotypic susceptible against colistin even though resistant determinant PmrB-65% was detected. Indicating that this truncation does not confer resistance or had too low concentration of bacteria in the suspension, causing to little growth.

One isolate showed phenotypic resistance to colistin, but no truncation was found. Indicating that there was too much growth, most likely resulting from high concentration of the bacteria in the suspension.

7. Conclusion

K. pneumoniae sensu stricto was found to be the most observed strain in this population. A high diversity of sequence types was detected with 378 different types, including three new STs. The most prevalent ST was ST107 (n=67). Among the 50 ESBL-harboring isolates, ST307 was the most prevalent, where it is associated with production of $bla_{\text{CTX-M-15}}$. $bla_{\text{CTX-M}}$ family was the dominant ESBL where $bla_{\text{CTX-M-15}}$ (n=34) was the main enzyme currently encountered in this population.

20.5% of this population have determinants encoding resistance against the investigated classes of antibiotics. 5 isolates were found with genotypic colistin resistance due to truncations of PmrB and MgrB. The level of multidrug resistance detected in this population is mainly resistant against aminoglycosides, fluoroquinolones and trimethoprim/ sulfamethoxazole, but also strongly associated with carriage of ESBL, predominantly $bla_{\text{CTX-M-15}}$. In this population, ST307 (n=11) and ST45 (n=5) were the sequence types predominantly associated with the MDR isolates. ST307 is linked to production of $bla_{\text{CTX-M-15}}$ and multidrug resistance.

Based on the results, genotype cannot predict phenotype despite the high level of matching results. However, detection of genotypic ESBL_{A}-genes showed phenotypic resistance against third-generation cephalosporins, indicating high probability to predict the phenotype based on genotypic resistance ESBL-genes.

8. Future perspectives

Due to the detection of $ESBL_A$-genes, predominantly *bla*$_{CTX\text{-}M\text{-}15}$, and multidrug resistance, further investigations regarding the characteristics of the bacteria should be investigated. ST307 is linked to production of *bla*$_{CTX\text{-}M\text{-}15}$ and multidrug resistance. Further investigation of this sequence type could be done. Closer study of *bla*$_{CTX\text{-}M\text{-}15}$ could be investigated to find out if there is a special plasmid connected to this $ESBL_A$-gene. Investigation of plasmids responsible for ESBL production and why and how they carry other determinants conferring resistance to other classes is also of interest.

9. List of References

[1] WHO. (2018, 7 December). *Antibiotic resistance* [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance.

[2] E. Martens and A. L.Demain, "An Overview of the Industrial Aspects of Antibiotic Discovery," Microbial ResourcesScience Direct: Academic Press, 2017, pp. 149-168. [Online]. Available: https://www.sciencedirect.com/science/article/pii/B9780128047651000072.

[3] WHO. (2018, 1. November). *Antimicrobial resistance* [Online]. Available: http://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance.

[4] K. L. Wyres and K. E. Holt, "Klebsiella pneumoniae Population Genomics and Antimicrobial-Resistant Clones," *Trends in Microbiology,* vol. Vol. 24, No. 12, pp. 944-956, 2016.

[5] K. E. Holt *et al.*, "Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in Klebsiella pneumoniae, an urgent threat to public health," July 7, 2015. Accessed on: 16.10.2018

[6] Illumina. (2018, 7 December). *A high-resolution view of the entire genome* [Online]. Available: https://www.illumina.com/techniques/sequencing/dna-sequencing/whole-genome-sequencing.html.

[7] R. Podschun and U. Ullmann, "Klebsiella spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors," vol. Vol. 11, No. 4, no. Clinical Microbiology Reviews, pp. 589-603, 16.10.2018 1998.

[8] M. K. Paczosa and J. Mecsasb, "Klebsiella pneumoniae: Going on the Offense with a Strong Defense," *Microbiology and Molecular Biology Reviews,* vol. Vol 80, No. 3, pp. 629-661, 2016.

[9] K. A. A. A. Rahim and A. M. A. Mohamed, "Prevalence of Extended Spectrum β-lactamase-Producing Klebsiella pneumoniae in Clinical Isolates," vol. 11. doi: 10.5812/jjm.17114 [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4332241/

[10] T. J. Kidd *et al.*, "A Klebsiella pneumoniae antibiotic resistance mechanism that subdues host defences and promotes virulence," vol. 4, pp. 430-447Accessed on: 2105.19. doi: 10.15252/emmm.201607336 [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5376759/

[11] ECDC, "Summary of the latest data on antibiotic resistance in the European Union," pp. 1-12, 2016.

[12] A. G. Elliott, D. Ganesamoorthy, L. Coin, M. A. Cooper, and M. D. Cao, "Complete Genome Sequence of Klebsiella quasipneumoniae subsp. similipneumoniae Strain ATCC 700603," vol. 4(3). doi: 10.1128/genomeA.00438-16 [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4882950/

[13] WHO. (2018, 31 October). *Antimicrobial Discovery and Development* [Online]. Available: http://www.who.int/drugresistance/Microbes_and_Antimicrobials/en/.

[14] S. B. Zaman, M. A. Hussain, R. Nye, V. Mehta, K. T. Mamun, and N. Hossain, "A Review on Antibiotic Resisitance: Alarm Bells are Ringing," vol. 9 (6), Accessed on: 31 October 2018. doi: 10.7759/cureus.1403 [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5573035/

[15] C. Hennequin and F. Robin, "Correlation between antimicrobial resistance and virulence in Klebsiella pneumoniae," *Eur J Clin Microbiol Infect Dis,* vol. 35, pp. 333-341, 2016.
[16] A. Wanger, R. S. P. Huang, J. K. Actor, V. Chavez, A. Wahed, and A. Dasgupta, "Antibiotics, Antimicrobial Resistance, Antibiotic Susceptibility Testing, and Therapeutic Drug Monitoring for Selected Drugs," Microbiology and Molecular Diagnosis in Pathology, 2017, pp. 119-153. [Online]. Available: https://www.sciencedirect.com/topics/neuroscience/beta-lactam-antibiotics.
[17] T. Loftsson, "β-Lactam Antibiotics," Drug Stability for Pharmaceutical Scientists, 2014, p. 170. [Online]. Available: https://www.sciencedirect.com/book/9780124115484/drug-stability-for-pharmaceutical-scientists.
[18] M.-P. Mingeot-Leclerc, Y. Glupczynski, and P. M. Tulkens, "Aminoglycosides: Activity and Resistance," vol. 43 (4), pp. 727-737Accessed on: 7 November 2018[Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC89199/
[19] J. E. Maddison, D. B. Church, and S. W. Page, "Antibacterial Drugs," Small Animal Clinical Pharmacology (Second Edition)Science Direct, 2008, pp. 148-185. [Online]. Available: https://www.sciencedirect.com/topics/medicine-and-dentistry/aminoglycoside.
[20] S. Swallow, "Flourine in Medical Chemistry," vol. 54, Progress in Medical ChemistryScience Direct, 2015. [Online]. Available: https://www.sciencedirect.com/topics/neuroscience/fluoroquinolone-antibiotics.
[21] D. Hooper, "Mechanisms of action of antimicrobials: focus on fluoroquinolones.," Accessed on: 8 November 2018. doi: 10.1086/319370 [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/11249823
[22] NCBI. (2005, 8 November). *Sulfamethoxazole And Trimethoprim* [Online]. Available: https://pubchem.ncbi.nlm.nih.gov/compound/CO-Trimoxazole - section=Top.
[23] T. R. Kemnic and M. Coleman, "Trimethoprim Sulfamethoxazole," [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK513232/
[24] L. T and D. A, "Colistin: an antibiotic and its role in multiresistant Gram-negative infections.," vol. 47(2), pp. 157-68[Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/26260559
[25] R. L. Nation and J. Li, "Colistin in the 21st Century," vol. 6, pp. 535-543Accessed on: 23.04.2019. doi: 10.1097/QCO.0b013e328332e672 [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2869076/
[26] B. AZ and S. K. H., "Colistin, mechanisms and prevalence of resistance.," pp. 21-707Accessed on: 23.04.2019. doi: 10.1185/03007995.2015.1018989 [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/25697677
[27] A. I and P. A, "A review on the current situation and challenges of colistin resistance in poultry production.," vol. 47(6), pp. 546-558. doi: 10.1080/03079457.2018.1524573 [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/30226397
[28] T. FC, "Mechanims of antimicrobial resistance in bacteria," vol. 34. doi: 10.1016/j.ajic.2006.05.219 [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/16813980
[29] C. L. Ventola, "The Antibiotic Resistance Crisis: Part 1: Causes and Threats," *P & T : a peer-reviewed journal for formulary management,*, vol. 40(4), pp. 277-283[Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378521/

[30] A. Brøgger. (2018). *Plasmid* [Online]. Available: https://snl.no/plasmid.
[31] R. H.-P. Dhillon and J. Clark, "ESBLs: A Clear and Present Danger?," Accessed on: 29 November 2018. doi: [10.1155/2012/625170] [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3135063/
[32] A. D. Russell, "Mechanisms of Bacterial Resistance to Antibiotics and Biocides," Progress in Medical Chemistry, D. R. Witty and B. Cox, Eds., Science Direct: Elsevier, 1998. [Online]. Available: https://www.sciencedirect.com/bookseries/progress-in-medicinal-chemistry.
[33] D. L. Paterson and R. A. Bonomo, "Extended-Spectrum B-Lactamases: a Clinical Update," vol. 4, pp. 657-686. doi: 10.1128/CMR.18.4.657-686.2005 [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1265908/
[34] M. B. Miller and P. H. Gilligan, "Laboratory Diagnosis and Therapy of Infectious Diseases," Principles and Practice of Pediatric Infectious Diseases (Fourth Edition), S. S. Long, Ed., Science Direct, 2012. [Online]. Available: https://www.sciencedirect.com/topics/medicine-and-dentistry/beta-lactamase.
[35] C. Giske *et al.*, "Redefining extended-spectrum beta-lactamases: balancing science and clinical need.," vol. 63 (1), 2008 Oct 28. doi: doi: 10.1093/jac/dkn444 [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/18957393
[36] P. A. Bradford, "Extended-Spectrum β-Lactamases in the 21st Century: Characterization, Epidemiology, and Detection of This Important Resistance Threat," vol. 14 (4), pp. 933-951Accessed on: 30 November 2018. doi: [10.1128/CMR.14.4.933-951.2001] [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC89009/
[37] L. S. Tzouvelekis, E. Tzelepi, P. T. Tassios, and N. J. Legakis, "CTX-M-type β-lactamases: an emerging group of extended-spectrum enzymes," vol. 14, no. 2, pp. 137-142Accessed on: 2 December 2018. doi: https://doi.org/10.1016/S0924-8579(99)00165-X [Online]. Available: https://www.sciencedirect.com/science/article/pii/S092485799900165X
[38] G. M. Rossolini, M. M. D'Andrea, and C. Mugnaioli, "The spread of CTX-M-type extended-spectrum β-lactamases," vol. 14, pp. 33-41Accessed on: 2 December 2018. doi: https://doi.org/10.1111/j.1469-0691.2007.01867.x [Online]. Available: https://www.sciencedirect.com/science/article/pii/S1198743X14604730
[39] ECDC. (2019). *How does antibiotic resistance spread?* [Online]. Available: https://ecdc.europa.eu/sites/portal/files/documents/how-does-antibiotic-resistance-spread.pdf.
[40] ECDC. Surveillance Atlas of Infectious Diseases [Online] Available: https://atlas.ecdc.europa.eu/public/index.aspx
[41] NORM/NORM-VET, "Usage of Antimicrobial Agents and Occurence of Antimicrobial Resistance in Norway. ," [Online]. Available: https://unn.no/Documents/Kompetansetjenester, -sentre og fagråd/NORM%20-%20Norsk%20overvåkingssystem%20for%20antibiotikaresistens%20hos%20mikrober/Rapporter/NORM_NORM-VET_2017.pdf
[42] M. Carter and J. Shieh, "Molecular Cloning and Recombinant DNA Technology," Guide to Research Techniques in Neurosciene Second Edition ed. Science Direct, 2015, pp. 219-237. [Online]. Available: https://www.sciencedirect.com/topics/medicine-and-dentistry/dna-sequencing.

[43] Illumina. (2019, 26 February). *Key differences between next-generation sequencing and Sanger sequencing* [Online]. Available: https://www.illumina.com/science/technology/next-generation-sequencing/ngs-vs-sanger-sequencing.html.
[44] Illumina, "Illumina Sequencing by Synthesis," ed. Youtube, 2016.
[45] Illumina, "An introduction to Next-Generation Sequencing Technology," Accessed on: 11 February 2019[Online]. Available: https://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf
[46] Illumina. (2015, 7 December). *Nextera XT Library Prep: Tips and Troubleshooting* [Online]. Available: https://www.illumina.com/content/dam/illumina-marketing/documents/products/technotes/nextera-xt-troubleshooting-technical-note.pdf.
[47] P. Albertus, "Illumina NGS Technology," Accessed on: 9 December 2018[Online]. Available: http://www.cbs.dtu.dk/courses/27008/NGS_mikrobiologer/slides/ILLUMINA.pdf
[48] K. Syal *et al.*, "Current and emerging techniques for antibiotic susceptibility tests." doi: 10.7150/thno.19217 [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5479269/
[49] T. D. Systems, "SENSITITRE® SUSCEPTIBILITY PLATES and JustOne® strips FOR CLINICAL FASTIDIOUS AND NON-FASTIDIOUS ORGANISMS," vol. 1.8, [Online]. Available: http://trekds.com/techDocs/techInsert/012-RESEARCH-GB-V1.8-CID8783.pdf
[50] Sensititre, "Sensititre NONAG5," Accessed on: 28 February 2019[Online]. Available: http://trekds.com/techDocs/panelLayout/NONAG5_170214.pdf
[51] Sensititre, "Sensititre NONAG4," Accessed on: 28 February 2019[Online]. Available: http://trekds.com/techDocs/panelLayout/NONAG4_170214.pdf
[52] Illumina. (2019, 25 February). *See what's possible with the MiSeq System* [Online]. Available: https://www.illumina.com/systems/sequencing-platforms/miseq.html.
[53] Illumina, "Hamilton Microlab STAR Reference Guide," Accessed on: 7 November 2018[Online]. Available: https://support.illumina.com/downloads/hamilton-ml-star-reference-guide.html
[54] L. Science. (2018, 7 November). [Online]. Available: https://www.lifescience.roche.com/en_no/products/magna-pure-96-instrument-382411-1.html - overview.
[55] Tecan. (2019, 25 February). *Spark* [Online]. Available: https://lifesciences.tecan.com/multimode-plate-reader?p=tab--2.
[56] ThermoFisher. (2019, 25 February). *Sensititre™ Nephelometer* [Online]. Available: https://www.thermofisher.com/order/catalog/product/V3011.
[57] T. Scientific. (2019, 15 February). *Sensititre AIM™ Automated Inoculation Delivery System* [Online]. Available: https://www.thermofisher.com/order/catalog/product/V3020.
[58] ThermoFisher. (2019, 25 February). *Sensititre™ Vizion™ Digital MIC Viewing System* [Online]. Available: https://www.thermofisher.com/order/catalog/product/V2020-SYS-LIS.
[59] ThermoFisher. (2019, 25 February). *Sensititre™ SWIN™ Software System* [Online]. Available: https://www.thermofisher.com/order/catalog/product/SW120.
[60] ThermoFisher. *Quant-iT™ dsDNA Assay Kit, high sensitivity*.

[61] Illumina. (26 November). *Nextera XT DNA Library Preparation Kit* [Online]. Available: https://www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/nextera-xt-dna.html.
[62] Illumina. *PhiX Control V3* [Online]. Available: https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/phix-control-v3.html.
[63] Illumina. (2019, 13 February). *MiSeq Reagent Kit v3* [Online]. Available: https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/miseq-reagent-kit-v3.html.
[64] S. J. Karcher, "TRANSPOSON MUTAGENESIS OF Escherichia coli," Molecular Biology, 1995, pp. 1-44. [Online]. Available: https://www.sciencedirect.com/topics/immunology-and-microbiology/inoculation-loop.
[65] Roche, "MagNA Pure 96 DNA and Viral NA Small Volume Kit," vol. 7, [Online]. Available: https://www.n-genetics.com/products/1295/1023/16530.pdf
[66] ThermoFisher. (2019). *Quant-iT dsDNA Assay Kit, High Sensitivity* [Online]. Available: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0002497_Quant_iT_dsDNA_Assay_Kit_HS_QR.pdf&title=UXVpY2sgUmVmOiBRdWFudC1pVCBkc0ROQSBBc3NheSBLaXQsIEhpZ2ggU2Vuc2l0aXZpdHk=.
[67] Illumina, "Nextera XT DNA Library Prep Kit," vol. 3, [Online]. Available: https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera-xt/nextera-xt-library-prep-reference-guide-15031942-03.pdf
[68] A. Technologies, " Agilent High Sensitivity DNA Kit Guide," [Online]. Available: https://www.agilent.com/cs/library/usermanuals/Public/G2938-90321_SensitivityDNA_KG_EN.pdf
[69] Illumina. (2018, 4 December). *Specifications for the MiSeq System* [Online]. Available: https://www.illumina.com/systems/sequencing-platforms/miseq/specifications.html.
[70] BaseSpace. (9 December). *Understanding Run Charts in BaseSpace Sequence Hub* [Online]. Available: https://help.basespace.illumina.com/articles/descriptive/runs-charts/.
[71] GitHub. (2019, 18 Februrary). *FastQC* [Online]. Available: https://github.com/s-andrews/FastQC.
[72] B. Bioinformatics. (9 December). *FastQC* [Online]. Available: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/.
[73] GitHub. (2019, 18 February). *MultiQC* [Online]. Available: https://github.com/ewels/MultiQC.
[74] P. Ewels, M. Magnusson, S. Lundin, and M. Käller. (2016, 9 December). *MultiQC* [Online]. Available: https://multiqc.info/.
[75] F. Krueger. Trim Galore [Online] Available: http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
[76] Illumina. (2018). *Assembling novel genomes* [Online]. Available: https://www.illumina.com/techniques/sequencing/dna-sequencing/whole-genome-sequencing/de-novo-sequencing.html.

[77] GitHub. (2019, 13 February). *Unicycler* [Online]. Available: https://github.com/rrwick/Unicycler.
[78] R. R. Wick, L. M. Judd, C. L. Gorrie, and K. E. Holt, "Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads," vol. 2, Accessed on: 13 February 2019. doi: https://doi.org/10.1371/journal.pcbi.1005595 [Online]. Available: https://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1005595
[79] GitHub. (2019, 18 February). *SPAdes* [Online]. Available: https://github.com/ablab/spades.
[80] B. J. Walker *et al.*, "Pilon: An Integrated Tool for Comprehensive Microbial Variant Detection and Genome Assembly Improvement," vol. 11. doi: https://doi.org/10.1371/journal.pone.0112963 [Online]. Available: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0112963
[81] A. Gurevich, V. Saveliev, N. Vyahhi, and G. Tesler. (2013, 9 December). *QUAST: quality assessment tool for genome assemblies* [Online]. Available: http://bioinf.spbau.ru/quast.
[82] GitHub. (2019, 15 February). *Quast* [Online]. Available: https://github.com/ablab/quast.
[83] M. Hetland. (2019, 21.05.19). *BAM_coverage* [Online]. Available: https://benchling.com/s/etr-unyiSkmyzfMs6Q3UXiRc/edit.
[84] GitHub. (2019). *Pickard Toolkit* [Online]. Available: https://github.com/broadinstitute/picard.
[85] GitHub. (2019). *SAMtools* [Online]. Available: https://github.com/samtools/samtools.
[86] GitHub. (2019, 18 February). *RedDog* [Online]. Available: https://github.com/katholt/RedDog.
[87] D. J. Edwards, B. J. Pope, and K. E. Holt. (2015). *RedDog: comparative analysis pipeline for large numbers of bacterial isolates using high-throughput sequences.* [Online]. Available: https://github.com/katholt/RedDog/wiki/1.-Instruction-Manual.
[88] Github. (2019, 18 February). *Bowtie2* [Online]. Available: https://github.com/BenLangmead/bowtie2.
[89] GitHub. (2019, 18 February). *SAMtools* [Online]. Available: https://github.com/samtools/samtools.
[90] GitHub. (2019, 18 February). *Kleborate* [Online]. Available: https://github.com/katholt/Kleborate.
[91] GitHub. (2019). *mashtree* [Online]. Available: https://github.com/lskatz/mashtree.
[92] NCBI. BLAST [Online] Available: https://blast.ncbi.nlm.nih.gov/Blast.cgi
[93] NCBI. (2019). *Klebsiella pneumoniae subsp. pneumoniae HS11286 chromosome, complete genome* [Online]. Available: https://www.ncbi.nlm.nih.gov/nuccore/NC_016845.1?report=graph.
[94] NCBI. (2019). *Klebsiella pneumoniae* [Online]. Available: https://www.ncbi.nlm.nih.gov/genome/?term=Klebsiella%20pneumoniae%5bOrganism%5d&cmd=DetailsSearch.
[95] Illumina. (2014, 09.06). *De Novo Assembly of Bacterial Genomes* [Online]. Available: https://www.illumina.com/content/dam/illumina-marketing/documents/products/appnotes/appnote-nextera-mate-pair-bacteria.pdf.
[96] C. Rodrigues, V. Passet, A. Rakotondrasoa, and S. Brisse, "Identification of Klebsiella pneumoniae, Klebsiella quasipneumoniae, Klebsiella variicola and Related Phylogroups

by MALDI-TOF Mass Spectrometry," vol. 9, p. 3000. doi: 10.3389/fmicb.2018.03000 [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6294014/

[97] K. L. Wyres *et al.*, "Emergence and rapid global dissemination of CTX-M-15-associated Klebsiella pneumoniae strain ST307," *Journal of Antimicrobial Chemotherapy,* vol. 74, pp. 577-581, 2019.

[98] R. Marando *et al.*, "Predictors of the extended-spectrum-beta lactamases producing Enterobacteriaceae neonatal sepsis at a tertiary hospital, Tanzania," vol. 308, no. 7, pp. 803-811. doi: https://doi.org/10.1016/j.ijmm.2018.06.012 [Online]. Available: https://www.sciencedirect.com/science/article/pii/S1438422118301759

[99] NORM. (2019, 4.06). *NORM-atlas* [Online]. Available: https://norm-atlas.no/sites/default/files/atlas/files/88/atlas.html.

[100] MatPrat. (2019, 13.06.2019). *LAVT ANTIBIOTIKABRUK I NORSK HUSDYRHOLD* [Online]. Available: https://www.matprat.no/artikler/matproduksjon/lavt-antibiotikabruk-i-norsk-husdyrhold/.

[101] X.-h. Hou, X.-y. Song, X.-b. Ma, S.-y. Zhang, and J.-q. Zhang, "Molecular characterization of multidrug-resistant Klebsiella pneumoniae isolates," vol. 46(3), pp. 759-768. doi: 10.1590/S1517-838246320140138 [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4568872/

[102] C. F *et al.*, "Impact of the ST101 clone on fatality among patients with colistin-resistant Klebsiella pneumoniae infection.," vol. 73(5), pp. 1235-1241. doi: 10.1093/jac/dkx532 [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/29415120

[103] C. Shankar, S. Kumar, M. Venkatesan, and B. Veeraraghavan, "Emergence of ST147 Klebsiella pneumoniae carrying blaNDM-7 on IncA/C2 with ompK35 and ompK36 mutations in India." doi: https://doi.org/10.1016/j.jiph.2019.03.023 [Online]. Available: https://www.sciencedirect.com/science/article/pii/S1876034119301339

[104] C. A. Bidewell *et al.*, "Emergence of Klebsiella pneumoniae subspecies pneumoniae as a cause of septicaemia in pigs in England," vol. 13(2). doi: 10.1371/journal.pone.0191958 [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5823397/

[105] K. J. Shaw, P. N. Rather, R. S. Hare, and G. H. Miller, "Molecular Genetics of Aminoglycoside Resistance Genes and Familial Relationships of the Aminoglycoside-Modifying Enzymes," *American Society for Microbiology,* vol. 57(1), pp. 138-163, 1993.

[106] J. G. Frye and C. R. Jackson, "Genetic mechanisms of antimicrobial resistance identified in Salmonella enterica, Escherichia coli, and Enteroccocus spp. isolated from U.S. food animals," vol. 4, p. 135. doi: 10.3389/fmicb.2013.00135 [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3661942/

[107] V. MW, H. SS, W. M, J. GA, H. DC, and B. JS, "Structure of QnrB1, a plasmid-mediated fluoroquinolone resistance factor.," vol. 286(28). doi: 10.1074/jbc.M111.226936 [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/21597116

Appendix A

Nextera XT library preparation for Illuminas MiSeq performed using Hamilton.

Tagmentation:

Hamilton performed following:

1. Following volumes were added to a Hard-Shell skirted PCR plate:

- 10 µl Tagment DNA buffer (TD)

- 5 µl normalized gDNA (0.2 ng/µl)

Pipetted to mix.

2. 5 µl Amplicon Tagment Mix (ATM) was added to each well on the PCR plate. Pipetted to mix.

3. The plate was centrifuged at 280 x g at 20 ^{o}C for 1 minute.

4. The plate was placed on the preprogrammed thermal cycler:

- 55^{o}C for 5 minutes, hold at 10^{o}C

5. 5 µl Neutralize Tagment Buffer (NT) was added to each well. Pipetted to mix.

6. The plate was and centrifuged at 280 x g at 20 ^{o}C for 1 minute before incubated at room temperature for 5 minutes.

Library Amplification:

Hamilton performed following:

1. The index adapters 1 (i7) were arranged in the columns 1-4 while the index 2 (i5) adapters were arranged in the rows A-H.

2. Pipettes were used to add 5 µl of each index 1 (i7) down each column.

3. Pipettes were used to add 5 µl of each index 2 (i5) across each row.

4. 5 µl of NPM were added to each well containing index adapters. Pipettes was used to mix.

6. The plate was centrifuged at 280 x g at 20^{o}C for 1 minute. Then, the plate was placed in the programmed thermal cycler and PCR program was runned.

PCR program on thermal cycler:

- 72°C for 3 minutes
- 95°C for 30 seconds
- 12 cycles of:
    - 95°C for 10 seconds
    - 55°C for 30 seconds
    - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 10°C

Clean Up Libraries:

Hamilton performed following:

1. The plate was centrifuged at 280 x g at 20°C for 1 minute.
2. 50 µl of PCR product from each well were transferred to a new midi plate.
3. 30 µl AMPure XP beads was added to each well.
4. The plate was put on the shaker for 2 minutes at 1800 rpm.
5. The plate was incubated at room temperature for 5 minutes.
6. The plate was placed on the magnetic stand for 2 minutes, until the liquid was clear.
7. All supernatant was removed and discarded from each well.
8. Following wash was performed two times:
    a. 200 µl of fresh 80% EtOH was added to each well.
    b. The plate was incubated on the magnetic stand for 30 seconds.
    c. All supernatant was removed and discarded.
9. The plate was put on the magnetic stand for 15 minutes to air-dry.
10. 52,5 µl RSB was added to each well.
11. The plate was put on the shaker for 2 minutes at 1800 rpm. Followed by incubation at room temperature for 2 minutes.
12. The plate was placed on the magnetic stand for 2 minutes until the liquid was clear.
13. 50 µl of supernatant was transferred to a new Hard-Shell PCR plate.

Normalize libraries:

Hamilton performed following:

1. 20 µl of the supernatant from the Hard-Shell PCR plate was transferred to a new midi plate.
2. 45 µl of combined LNA1 and LNB1 was added to each well.
3. The plate was put on the shaker for 30 minutes at 1800 rpm.
4. The plate was put on the magnetic stand for 2 minutes until the liquid was clear.
5. All supernatant from each well was removed and discarded.
6. Following wash was performed two times:
    a. 45 µl of LNW1 was added to each well.
    b. The plate was put on the shaker for 5 minutes at 1800 rpm.
    c. The plate was incubated on the magnetic stand for 30 seconds.
    d. All supernatant was removed and discarded.
7. 30 µl 0.1 M NaOH was added to each well.
8. The plate was put on the shaker for 5 minutes at 1800 rpm. Meanwhile, 30 µl LNS1 was added to each well of a new midi plate.
9. The plate on the shaker was placed on the magnetic stand for 2 minutes until the liquid was clear.
10. The supernatant was transferred from the midi plate to the new midi plate containing LNS1.
11. The plate was centrifuged at 1000 x g for 1 minute.

Pool libraries:

Hamilton performed following:

1. The plate was centrifuged at 1000 x g for 1 minute.
2. 5 µl of each library from the midi plate was added to an Eppendorf tube.

Appendix B

Re-sequenced isolates.

Table B.1: Re-sequenced isolates due to poor quality scores.

Isolates for resequencing	Poor quality – reason for resequencing	Results after resequencing
NK-43	No contigs over 10 000 bp, total length= 7279	Contig = 653126 bp, Total length = 5259112 bp
NK-44	Contigs = 1001, L50 = 53	Contigs = 55, L50 = 10
NK-45	Contigs = 1102	Contigs = 56
NK-46	ST107 missing wzi gene	wzi74
NK-47	Incomplete ESBL-gene: SHV-2?	BLAST = SHV-2, 100% coverage and 100% sequence identity to accession number: MF4029301.1
NK-48	L50 = 51	L50 = 7
NK-49	Incomplete ESBL-gene: SHV-12*?	BLAST = SHV-2a, 100% coverage and 100% sequence identity to accession number: MF402908.1
NK-34	L50 = 60	L50 = 8
NK-50	Contigs = 404, L50 = 51	Contigs = 81, L50 = 7
NK-11	L50 = 48	L50 = 11
NK-51	L50 = 51, GC = 58.03	L50 = 11, GC = 57,57%
NK-52	No ST given and capsule wzi missing	New ST provided by the Institute Pasteur = ST4009
NK-53	No ST given and capsule wzi missing	New ST provided by the Institute Pasteur = ST4010
NK-54	No ST given and capsule wzi missing	New ST provided by the Institute Pasteur = ST4011

NK-55	N50<100k	N50 = 79700, included due to other good quality metrics
NK-56	Low N50	N50 = 269061
NK-57	L50 = 41	L50 = 16
NK-58	L50 = 41, largest contig 100574	L50 = 9, largest contig = 796026
NK-59	ST107 missing wzi gene	wzi74
NK-60	N50<100k	N50 = 94764, included due to other good quality metrics

Appendix C

Table C.1 and C.2 shows unpublished work from NORKAB and hereof explains why SHV-13* and SHV-101* were excluded as $ESBL_A$-genes.

4 other SHV-13* was not included based on the similarities with the isolates in table C.1.

Table C.1: *Phenotypic testes isolates regarding imprecise match of SHV-13*. Unpublished data from NORKAB.*

SHV-13*	NK-68	NK-69	NK-70
Trimetoprim-sulfa	-	S	S
Ciprofloxacin	S	S	S
Cefuroxime	S	S	S
Cefotaxime	S	S	S
Ceftazidime	S	S	S
Gentamicin	S	S	S
Meropenem	S	S	S
Piperacillin-tazobactam	S	S	S
Phenotypic ESBL	-	-	-

Table C.2: *Phenotypic testes isolates regarding imprecise match of SHV-101*. Unpublished data from NORKAB.*

SHV-101*	NK-61	NK-62	NK-63	NK-64	NK-65	NK-66[1]	NK-67
Trimethoprim-sulfa	S	R	R	S	R	-	R
Ciprofloxacin	S	S	S	S	S	-	S
Cefuroxime	S	S	S	S	S	-	S
Cefotaxime	S	S	S	S	S	-	S
Ceftazidime	S	S	S	S	S	-	S
Gentamicin	S	S	S	S	S	-	S
Meropenem	S	S	S	S	S	-	S
Piperacillin-tazobactam SIR	S	S	S	S	S	-	S
Phenotypic ESBL	-	-	-	-	-	-	-

[1]NK-66 was identical to the other SHV-101* and hereby not included as an ESBL-gene.

Appendix D

Complete tables showing the results from AST compared to genotypic resistance determinants and the MIC-values for the antibiotics used.

Table D.1: *Results from antimicrobial susceptibility testing, resistance genes found and a comparison of these. Regarding β-lactams.*

Isolate #	Micro broth dilution		Resistance determinants	Comparison
	Beta-lactam			
	3rd generation-cephalosporins			
	Ceftazidime	Cefotaxime		
NK-1	I	R	CTX-M-1	Partial match
NK-2	R	R	CTX-M-15	Match
NK-3	R	R	CTX-M-15	Match
NK-4	R	R	CTX-M-15	Match
NK-5	R	R	CTX-M-15	Match
NK-6	R	R	CTX-M-15	Match
NK-7	S	S	-	Match
NK-8	R	R	CTX-M-15	Match
NK-9	R	R	CTX-M-15	Match
NK-10	R	R	CTX-M-15	Match
NK-11	R	R	CTX-M-15	Match
NK-12	R	R	SHV-11;CTX-M-15	Match
NK-13	R	R	CTX-M-15	Match
NK-14	R	R	CTX-M-15	Match
NK-15	S	S	-	Match
NK-16	R	R	CTX-M-15	Match
NK-17	R	R	CTX-M-15	Match
NK-18	R	R	CTX-M-15	Match
NK-19	R	R	CTX-M-14	Match
NK-20	R	R	CTX-M-15	Match
NK-21	R	R	CTX-M-15	Match
NK-22	R	R	CTX-M-15	Match
NK-23	R	R	CTX-M-15	Match
NK-24	R	R	CTX-M-14	Match
NK-25	R	R	CTX-M-15	Match
NK-26	R	R	CTX-M-15	Match

NK-27	R	R	CTX-M-15	Match
NK-28	R	R	CTX-M-15	Match
NK-29	R	R	CTX-M-15	Match
NK-30	R	R	CTX-M-15	Match
NK-31	R	R	SHV-1;CTX-M-15	Match
NK-32	R	R	CTX-M-15	Match
NK-33	S	S	-	Match
NK-34	R	R	CTX-M-15	Match
NK-35	R	R	CTX-M-15	Match
NK-36	R	R	-	No match
NK-37	R	R	CTX-M-15	Match
NK-38	R	R	CTX-M-15	Match
NK-39	R	R	CTX-M-15	Match
NK-40	R	R	CTX-M-3	Match
NK-41	R	R	CTX-M-15	Match

Table D.2: *Results from antimicrobial susceptibility testing, resistance genes found and a comparison of these. Regarding aminoglycosides.*

Isolate #	Micro broth dilution			Resistance determinants	Comparison
	Aminoglycosides				
	Amikacin	Gentamicin	Tobramycin		
NK-1	S	S	S	-	Match
NK-2	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-3	I	S	R	StrB;StrA*	No match
NK-4	S	S	S	AadA2*;StrB*;StrA*;Aph3-Ia*	Match[1]
NK-5	S	S	S	StrB;StrA*	Match
NK-6	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-7	S	S	S	-	Match
NK-8	S	S	S	-	Match
NK-9	S	S	S	AadA2*;StrB*;StrA*;Aph3-Ia*	Match[1]
NK-10	S	S	S	StrB*;StrA*	Match
NK-11	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-12	S	S	S	-	Match
NK-13	S	S	S	StrB;StrA*	Match
NK-14	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-15	S	S	S	-	Match
NK-16	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-17	S	S	S	-	Match
NK-18	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-19	S	R	R	Aac3-IId*;StrB*?;StrA*	Match[1]
NK-20	S	S	S	StrB;StrA*	Match
NK-21	S	S	S	StrB;StrA*	Match
NK-22	I	S	R	StrB;StrA*	No match
NK-23	S	R	R	Aac3-IIa*	Match[1]
NK-24	S	R	R	StrB*?;StrA*;Aac3-IId*	Match[1]
NK-25	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-26	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-27	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-28	S	S	S	StrB;StrA*	Match

NK-29	I	S	R	StrB;StrA*	No match
NK-30	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-31	S	R	R	StrB*;StrA*;AadA2*;Aac3-IIa*	Match[1]
NK-32	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-33	S	S	S	-	Match
NK-34	I	S	R	StrB;StrA*	No match
NK-35	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-36	R	R	R	-	No match
NK-37	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-38	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-39	S	R	R	StrB;StrA*;Aac3-IIa*	Match[1]
NK-40	S	S	S	-	Match
NK-41	S	S	S	StrB;StrA*	Match

[1]Indicated that the resistance gene was present

[*]Indicates imprecise allele match

[?]Indicates that the length of match is less than the length of the reported allele

Table D.3: *Results from antimicrobial susceptibility testing, resistance genes found and a comparison of these. Regarding fluoroquinolones.*

Isolate #	Micro broth dilution Fluoroquinolones	Resistance determinants	Comparison
NK-1	S	-	Match
NK-2	R	QnrB1?	Match[1]
NK-3	R	QnrB1?;GyrA-83I;ParC-80I	Match[1]
NK-4	R	Qnr-S1	Match
NK-5	R	QnrB1?	Match[1]
NK-6	R	QnrB1?	Match[1]
NK-7	S	-	Match
NK-8	R	QnrB1?	Match[1]
NK-9	R	Qnr-S1	Match
NK-10	R	QnrB1?	Match[1]
NK-11	R	QnrB1?;GyrA-83I;ParC-80I	Match[1]
NK-12	S	-	Match
NK-13	R	QnrB1?	Match[1]
NK-14	R	QnrB1?;GyrA-83I;ParC-80I	Match[1]
NK-15	S	-	Match
NK-16	R	GyrA-83I;ParC-80I	Match[1]
NK-17	R	Qnr-S1	Match
NK-18	R	QnrB1?;GyrA-83I;ParC-80I	Match[1]
NK-19	R	Qnr-S1	Match
NK-20	R	QnrB1?	Match[1]
NK-21	R	Qnr-S1;GyrA-83I;ParC-80I	Match
NK-22	R	QnrB1?	Match[1]
NK-23	R	GyrA-83Y;GyrA-87N;ParC-80I	Match
NK-24	R	Qnr-S1	Match
NK-25	R	QnrB1?	Match[1]
NK-26	R	QnrB1?;GyrA-83I;ParC-80I	Match[1]
NK-27	R	QnrB1?	Match[1]
NK-28	R	Qnr-S1	Match
NK-29	R	QnrB1?	Match[1]

NK-30	R	QnrB1?;GyrA-83I;ParC-80I	Match[1]
NK-31	R	Qnr-S1	Match
NK-32	R	QnrB1?;GyrA-83I;ParC-80I	Match[1]
NK-33	S	-	Match
NK-34	R	QnrB1?	Match[1]
NK-35	R	QnrB1?	Match[1]
NK-36	R	-	No match
NK-37	R	QnrB1?;GyrA-83I;ParC-80I	Match[1]
NK-38	R	GyrA-83I;ParC-80I	Match
NK-39	R	QnrB1?;GyrA-83I;ParC-80I	Match[1]
NK-40	S	-	Match
NK-41	S	QnrB1?	No match[1]

[1]Indicated that resistance gene was present

[?]Indicated that the length of match is less than the length of the reported allele

Table D.4: *Results from antimicrobial susceptibility testing, resistance genes found and a comparison of these. Regarding trimethoprim/ sulfamethoxazole.*

Isolate #	Micro broth dilution Trimethoprim/ sulfamethoxazole	Resistance determinants	Comparison
NK-1	S	SulII	Partial match
NK-2	R	SulII and DfrA14	Match
NK-3	R	SulII and DfrA14	Match
NK-4	R	SulI;SulII and DfrA12	Match
NK-5	R	SulII* and DfrA14	Match
NK-6	R	SulII and DfrA14	Match
NK-7	S	-	Match
NK-8	S	-	Match
NK-9	R	SulI;SulII and DfrA12	Match
NK-10	R	SulII and DfrA14	Match
NK-11	R	SulII and DfrA14	Match
NK-12	S	-	Match
NK-13	R	SulII* and DfrA14	Match[1]
NK-14	R	SulII and DfrA14	Match
NK-15	S	-	Match
NK-16	R	SulII and DfrA14	Match
NK-17	S	-	Match
NK-18	R	SulII and DfrA14	Match
NK-19	R	SulII and DfrA14	Match
NK-20	R	SulII and DfrA14	Match
NK-21	R	SulII and DfrA14	Match
NK-22	R	SulII and DfrA14	Match
NK-23	R	DfrA14	Partial match
NK-24	R	SulII and DfrA14	Match
NK-25	R	SulII and DfrA14	Match
NK-26	R	SulII and DfrA14	Match
NK-27	R	SulII and DfrA14	Match
NK-28	R	SulII and DfrA14	Match

NK-29	R	SulII and DfrA14	Match
NK-30	R	SulII and DfrA14	Match
NK-31	R	SulII;SulI and DfrA12	Match
NK-32	R	SulII and DfrA14	Match
NK-33	S	-	Match
NK-34	R	SulII and DfrA14	Match
NK-35	R	SulII and DfrA14	Match
NK-36	S	-	Match
NK-37	R	SulII and DfrA14	Match
NK-38	R	SulII and DfrA14	Match
NK-39	R	SulII and DfrA14	Match
NK-40	R	DfrA14	Partial match
NK-41	R	SulII and DfrA14	Match

[1]Indicated that resistance gene was present

[*]Indicates imprecise allele match

Table D.5: *Results from antimicrobial susceptibility testing, resistance genes found and a comparison of these. Regarding colistin.*

Isolate #	Micro broth dilution Colistin	Resistance determinants	Comparison
NK-1	S	-	Match
NK-2	S	-	Match
NK-3	R	-	No match
NK-4	S	-	Match
NK-5	S	-	Match
NK-6	S	-	Match
NK-7	S	-	Match
NK-8	S	-	Match
NK-9	S	-	Match
NK-10	S	-	Match
NK-11	S	-	Match
NK-12	S	-	Match
NK-13	S	-	Match
NK-14	S	-	Match
NK-15	S	-	Match
NK-16	S	-	Match
NK-17	S	-	Match
NK-18	S	-	Match
NK-19	S	-	Match
NK-20	S	-	Match
NK-21	S	-	Match
NK-22	S	-	Match
NK-23	R	MgrB-89%	Match
NK-24	S	-	Match
NK-25	S	-	Match
NK-26	S	-	Match
NK-27	S	-	Match
NK-28	S	-	Match
NK-29	S	-	Match
NK-30	S	-	Match
NK-31	S	-	Match
NK-32	S	-	Match

NK-33	S	-	Match
NK-34	S	-	Match
NK-35	S	-	Match
NK-36	R	PmrB-65%	Match
NK-37	S	-	Match
NK-38	S	-	Match
NK-39	S	-	Match
NK-40	S	-	Match
NK-41	S	-	Match

Table D.6: *MIC-values for micro broth dilution.*

Antibiotics	MIC breakpoints (mg/L):	
	S ≤	R >
Amikacin	8	16
Gentamicin	2	4
Tobramycin	2	4
Colistin	2	2
Ceftazidime	1	4
Cefotaxime	1	2
Ciprofloxacin	0,25	0,5
Trimethoprim/sulfamethoxazole	2	4